IDENTIFYING MECHANISMS OF PYRETHROID RESISTANCE IN THE NAVEL ORANGEWORM AND NOVEL METHODS OF CONTROL

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Entomology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

The navel orangeworm (*Amyelois transitella*) (Walker) is the most important economic pest of almonds and pistachios in California orchards. Increasing demand for these commodities has resulted in acreage expansions and substantial increases in the application of insecticides to reduce damage by *A. transitella*. Pyrethroid insecticides have historically been the most heavily applied insecticides registered for *A. transitella* control because of their efficacy against all life stages, broad-spectrum activity in orchards, and significantly reduced costs relative to other insecticides. The first incidence of pyrethroid resistance in *A. transitella* occurred in Kern County almond orchards in 2013, and the initial mechanism reported suggested overexpression of cytochrome P450 monooxygenases (P450s) and carboxylesterases (COEs).

In the first chapter of my dissertation, I investigated the role of all P450s in the CYP3 and CYP4 clans associated with metabolism of xenobiotics in *A. transitella* through a comparative analysis using a susceptible population collected from Madera County almond orchards in 2016 (ALM) with a pyrethroid-resistant population (R347) collected from Kern County orchards in 2016. The objective of this research was to examine changes in gene expression in the ALM and R347 populations of *A. transitella* in order to identify P450s induced by bifenthrin and associated with pyrethroid resistance. I extracted RNA from midguts of fifth instar larvae that fed on artificial diets with and without bifenthrin, and I carried out quantitative real-time PCR (qRT-PCR) analyses of 65 P450s in the CYP3 and CYP4 clans of *A. transitella*. I identified only two P450s induced by bifenthrin, both of which occurred in the ALM population. Nine P450s were overexpressed in R347 larvae that fed on control diets, which suggested constitutive overexpression as a potential mechanism of pyrethroid resistance in this population. Among the

nine P450s overexpressed in the resistant population, two were associated with the synthesis of cuticular hydrocarbons (CHCs) in the CYP4G subfamily, which has been linked to resistance in other insects by preventing or delaying passage of insecticides across the cuticle. CHCs were then extracted and quantified between the susceptible and resistant populations, and results confirmed that the resistant population produces more CHCs in eggs and adults. I carried out a series of bioassays of topical toxicity to determine if elevated CHCs in the resistant population contribute to differences in egg and larval mortality through bifenthrin sprays. R347 egg mortality was reduced at low bifenthrin concentrations, and more R347 larvae survived bifenthrin treatment when challenged with higher concentrations. Whether or not CHCs contribute to enhanced survival of R347 under field conditions remains an open question.

For the second chapter of my dissertation, I annotated the carboxylesterases (COEs) in the *A. transitella* genome. Insect COEs are involved in developmental and neurological processes, pheromone processing and degradation, and metabolism of xenobiotics. Insect COEs are classified into subfamilies that include alpha-esterases, juvenile hormone esterases, integument esterases, beta-esterases, acetylcholinesterases, gliotactins, glutactins, neuroligins, and neurotactins through phylogenetic and functional analyses. I discovered 64 total COEs in the *A. transitella* genome and placed them all into their corresponding subfamilies by constructing a phylogeny with *Bombyx mori*, *Plutella xylostella*, and *Trichoplusia ni*, which are the only lepidopterans with fully annotated COEs. I identified an expansion in the number of alpha-esterases in *A. transitella*, which is consistent with all other lepidopteran insects described to date. Among the alpha-esterases, there are two clades in my phylogeny with orthologs from each species that are likely noncatalytic, with functions that are potentially unique to Lepidoptera.

These findings can provide a foundation for future research on investigating COE involvement in resistance to insecticides.

The third chapter of my dissertation was based upon previous research that identified a selective sweep in the *A. transitella* genome delimited by a point mutation *kdr* in the *para* gene that alters the conformation of the voltage-gated sodium channel and confers resistance to pyrethroids. This mutation was identified in three separate populations from the San Joaquin Valley of California, two of which came from areas without any previously described pyrethroid resistance. Although the sweep was present in the reference genome population, the mutation in *para* was absent. I re-sequenced the *para* gene to confirm that the mutation was absent in the reference genome population and also sequenced a population collected from two counties in northern California where pyrethroid applications have historically been less intense in almond orchards. After unexpectedly detecting the resistance mutation in the northern populations, I investigated the history of insecticide use in Kern County, Madera County, Colusa County, and Yolo County, where these *A. transitella* individuals were collected, with emphasis on the pyrethroid use. The insecticide records maintained through the California Department of Pesticide Regulations revealed a surge in bifenthrin use from 2009 to 2013 throughout the state before the first reported case of resistance arose in Kern County almond orchards in 2013. The heavy use of bifenthrin may have resulted from patent expiration and the availability of alternative product forms. The number of trade name products containing bifenthrin increased from one in 2009 to thirteen by 2013 in statewide almond orchards and one to nine products in pistachio orchards during this time. Comparisons of bifenthrin use relative to all other pyrethroids by pounds applied from 2009 to 2017 revealed that Kern County and Madera County applications were similar to statewide use at approximately 70% of all pounds of pyrethroids

applied. Bifenthrin use was higher in Kern County pistachio orchards at 59.6% all of all pyrethroid pounds applied compared to 51.1% statewide and 45.8% in Madera County. Bifenthrin may have accelerated resistance acquisition in *A. transitella*, although this analysis of insecticide use was based on county pooled averages and did not account for site-specific applications. Site-specific pyrethroid use may have been a determining factor in the development of resistance in Kern County where as few as ten companies may control 75% of the almond acreage. Records of pyrethroid use for the northern counties of Colusa and Yolo revealed lower bifenthrin selection pressure from 2009 to 2013 and did not correlate with trends in Kern County, Madera County, and statewide use. I suggest a need to examine a broader range of populations to determine the spread of pyrethroid resistance resulting from the *kdr* mutation in the *para* gene.

In the fourth and final chapter of my dissertation, I describe a series of experiments aimed at determining the potential for agricultural adjuvants to synergize the toxicity of two diamide insecticides registered at the time for *A. transitella* control. Despite widespread adoption of insecticide sprays for *A. transitella* control, the potential toxicity of adjuvants applied in combination with insecticides is unknown. In these experiments, five adjuvants currently applied by growers to manage *A. transitella* (Cohere®, Dyne-Amic®, FastStrike®, Induce®, Latron B-1956®) were examined alone and in combination with two diamide insecticides registered for use in almond and pistachio orchards, chlorantraniliprole (Altacor®) and flubendiamide (Belt®). Toxicity of adjuvant and adjuvant-diamide combinations was assessed against *A. transitella* eggs and adults through a series of laboratory experiments involving a spray tower. A series of field trials tested adjuvant-diamide combinations using orchard air-blast sprayers against the same life stages. Laboratory exposure of eggs and adults demonstrated that all adjuvants were intrinsically toxic to *A. transitella* and that toxicity of adjuvants and adjuvant-diamide combinations varied

across life stages. Field experiments demonstrated that adjuvants affected the toxicity of insecticides sprayed for *A. transitella* control. This study examined an overlooked and vital component to insecticide applications in tree nuts and suggests adjuvant choice has the potential to improve insecticide performance for *A. transitella* control.

ACKNOWLEDGMENTS

There have been many kind and generous people that have helped me succeed obtaining a BS, MS, and PhD from the University of Illinois. First and foremost, I thank my advisor Dr. May Berenbaum for all the opportunities and guidance she has provided over the years and for teaching me how to grow as a scientist. I cannot think of a better collaborator than Dr. Joel Siegel, and I thank him for providing his navel orangeworm expertise and granting me permission to work in his laboratory in California. I thank Dr. Bettina Francis and Dr. Stewart Berlocher for their suggestions and feedback on my dissertation. I thank all Berenbaum laboratory members, especially Dr. Bernarda Calla for teaching me different molecular techniques in the laboratory, introducing me to genomics and annotations, and for her patience in answering all my questions. I thank Dr. Esther Ngumbi for allowing me to use and analyze her cuticular hydrocarbon data on navel orangeworm eggs and adults in Chapter 1 of this dissertation and for providing the methods in those experiments. Thank you to my undergraduate advisor Dr. Mary Schuler for taking a chance on me and guiding me through research on insecticide detoxification by cytochrome P450 monooxygenases. Thank you to Brad Higbee for the opportunity to collaborate and advance our knowledge on resistance in the navel orangeworm. I thank Dr. Spencer Walse, Matt Rodriguez, and Erik Rankel for their collaboration on adjuvant research. Thank you to Dr. Kimberly Steinmann for providing records requested from the California Department of Pesticide Regulations. This research would not have been possible without consistent support from the Almond Board of California and the California Pistachio Research Board. I thank all almond and pistachio growers that have funded and made this research possible. Thank you as well to the Illinois Graduate College for awarding me the

Dissertation Completion Fellowship. I thank Kim Leigh for all of her assistance over the years and for helping put this dissertation together. Of course, I never would have made it this far without the encouragement and support provided by my family. Finally, I thank all the friends I have made at Illinois over the years for all their assistance and many great memories including Nathalie Baena, Aron Katz, Sam Oehlert, and Charles Dean.

This dissertation is dedicated to my family for their continuous love and support throughout my graduate research

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CHAPTER 1

CANDIDATE CYTOCHROME P450 MONOOXYGENASES IN THE CYP3 AND CYP4 CLANS CONTRIBUTING TO BIFENTHRIN RESISTANCE IN THE NAVEL ORANGEWORM (*AMYELOIS TRANSITELLA*) (LEPIDOPTERA: PYRALIDAE)

INTRODUCTION

The acquisition of resistance to synthetic insecticides by agricultural pests presents a continous challenge to the development of sustainable management strategies (Brattsten *et al.* 1986). Cytochrome P450 monoxygenases (P450s) have been extensively investigated for their roles in xenobiotic metabolism in insects. P450s are Phase I detoxification enzymes that catalyze oxidation reactions involving both endogenous and exogenous compounds; these enzymes take part in insect development, communication, and detoxification (Li *et al.* 2007). The genetic diversity, broad substrate recognition, and catalytic versatility of P450s have enabled insect pests to acquire resistance to multiple classes of insecticides irrespective of modes of action through mechanisms including constitutive overexpression, mutations affecting substrate specificity, and induction through food sources (Li *et al.* 2007, Feyereisen 2011, Schuler and Berenbaum 2013).

The highly polyphagous navel orangeworm *Amyelois transitella* Walker (Lepidoptera: Pyralidae) is the most economically important pest of almonds and pistachios in California orchards. Larvae are internal feeders whose damage contributes not only to direct losses but also to indirect losses through contamination by aflatoxin-producing *Aspergillus* spp. (Palumbo *et al.* 2014). The high demand for tree nuts (> 7-billion-dollar industry for California almonds and pistachios) (ACP 2018, NASS 2018) has led to increased insecticide applications to reduce *A. transitella* damage to acceptable levels. Insecticides are sprayed for *A. transitella* control when

the hulls of almonds and pistachios split and are vulnerable to oviposition. The most heavily applied insecticides in almond and pistachio orchards are pyrethroids because of their broad-spectrum activity against additional orchard pests and their significantly lower costs relative to other insecticides (<USD$2.00-5.00 per treated acre) (Demkovich et al 2015a, CDPR 2016). *A. transitella* resistance to pyrethroids was first reported in 2013 in Kern County almond orchards. Studies with synergists suggested that resistance to pyrethroids in Kern County involved in part cytochrome P450 monooxygenase (P450) enzymes (Demkovich *et al.* 2015a). The use of a synergist, however, cannot isolate specific P450s directly involved in metabolism.

Insect P450s are divided into 4 clans: CYP2, CYP3, CYP4, and mitochondrial P450s (Yu *et al.* 2015). The functions of P450s in the CYP2 and mitochondrial clans are conserved among insect species and limited variability include biosynthesis and metabolism of steroid hormones (Feyereisen 2012). Thus, there is in gene number and amino acid sequence of CYP2 and mitochondrial P450s (Calla *et al.* 2017). P450s in the CYP3 and CYP4 clans, however, have proliferated as a result of duplication events enabling diversification and acquisition of new substrates (Berenbaum 2002). Clan 3 P450s have diversified into many xenobiotic-metabolizing families involved with insecticide resistance, including CYP6, CYP9, and CYP321 (Yang *et al.* 2006, Hu *et al.* 2014, Ishak *et al.* 2017). CYP6AB11 was the first P450 characterized in *A. transitella* in 2011. CYP6AB11 metabolizes imperatorin, a phytochemical present in some hostplants, when expressed in a baculoviral expression system; however, no metabolism of the pyrethroid insecticide examined in this study, alpha-cypermethrin, was detected in this system (Niu *et al.* 2011).

Although some Clan 4 members are capable of metabolizing xenobiotics, the role of CYP4 P450s in general is less well understood, with fewer examples connecting functions to

insecticide resistance (Feyereisen 2012). Recent studies have confirmed the participation of CYP4G P450s in the synthesis of cuticular hydrocarbons (CHCs) as a mechanism of resistance to insecticides via reduced penetrance (Balabanidou *et al.* 2016, Mishra *et al.* 2018, Chen *et al.* 2019). Insect CHCs comprise the epicuticular wax layer that functions as a barrier against desiccation in many insects and also serve as signaling compounds that provide species-, colony-, sex-, and task-specific cues (Blomquist and Bagnères 2010, Yu *et al.* 2016). CHCs contribute to insecticide resistance by acting as barriers that reduce the amount of insecticide reaching the target site (Balabanidou *et al.* 2016, Yahouédo *et al.* 2017). Qiu *et al.* (2012) demonstrated that CYP4G1 and CYP4G2 catalyze the final step of CHC synthesis in *Drosophila melanogaster* and *Musca domestica*, and overexpression of CYP4Gs has been linked to pyrethroid resistance in *Anopheles gambiae* (Ingham *et al.* 2014, Balabanidou *et al.* 2016, Yahouédo *et al.* 2017), *Musca domestica* (Højland and Kristensen 2017), *Triatoma infestans* (Calderón-Fernández *et al.* 2017), *Drosophila suzuki* (Mishra *et al.* 2018), *Blattella germanica* (Chen *et al.* 2019), and *Helicoverpa armigera* (Pittendrigh *et al.* 1997). To date, however, resistance resulting from enhanced CYP4G synthesis of CHCs has not been documented in any lepidopterans.

Access to the complete inventory of P450s in *A. transitella* presented an opportunity to identify P450s that metabolize bifenthrin and that contribute to pyrethroid resistance through comparative analysis with a susceptible population. Calla *et al.* (2017) annotated the P450s of *A. transitella* and established a complete CYPome containing 89 total P450s, with 65 in the CYP3 and CYP4 Clans. In this study, I conducted high-throughput quantitative real-time PCR (qRT-PCR) analysis of all P450s within the CYP3 and CYP4 clans from resistant and susceptible *A. transitella* strains to identify transcripts that are inducible by bifenthrin and potentially involved in resistance. I then examined cuticular hydrocarbon differences between these strains using a

quantitative GC/MS approach and also quantified bifenthrin resistance through feeding assays with first instar larvae and through spray application assays designed to compare egg mortality and larval contact toxicity between the two strains.

MATERIALS AND METHODS

Insects

A bifenthrin-susceptible strain (ALM) of *A. transitella* was collected from unharvested nuts (mummies) in Madera County almond orchards by Dr. Joel Siegel (USDA-ARS) in 2016. A bifenthrin-resistant strain (R347) of *A. transitella* were collected from almond mummies in Kern County orchards by Brad Higbee (Trécé) in 2016. Larvae collected directly from mummies in Madera orchards were shipped to Illinois. Eggs were sent to Illinois from resistant adults that were reared from larvae in mummies from Kern County orchards. *A. transitella* received at Illinois were initially reared on a wheat-bran based diet (Finney and Brinkman 1967) to adulthood in 500 ml Mason jars and maintained in an incubator set at 27 ± 4º C with a photoperiod of 16:8 (L:D). Adults were collected and placed in Mason jars with dry paper towels to serve as a substrate for oviposition. Eggs were collected every 48 h to provide hatching larvae for use in qRT-PCR experiments and for use directly and as larvae in bioassays.

Insect sample preparation for qRT-PCR experiments

All insects were reared on semi-synthetic artificial diet (Waldbauer *et al.* 1984) without insecticides until fifth instar. Within 24 h of molting into fifth instar, larvae were removed from the rearing diet and placed on the same type of artificial diet containing either 0.5 ppm bifenthrin or methanol as the control solvent. This bifenthrin concentration was the maximum level that

maintained consistent feeding by fifth instars after transfer. Six larvae from each strain fed on control diets containing 200 µl in 5 g of artificial diet, and six from each strain fed on 0.5 ppm bifenthrin in 5 g of diet for a total of 24 larvae. All individual diet cups for controls and bifenthrin treatments contained a single larva. After feeding for 48 h, midguts were dissected from all larvae and flash-frozen in liquid nitrogen.

Sample processing

RNA was extracted from each of the 24 samples using a Nucleospin® RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The RNA was quantitated with a Nanodrop spectrophotometer (ThermoFisher Scientific), and 1 µg of it was used to synthesize cDNA with Protoscript II kit (New England Biolab, Ipswich, MA USA). The cDNA for each of the 24 samples was tested by end-point PCR with primers specific for the *A.transitella* Actin-5 (NCBI Gene ID: LOC106142213) and evaluated in a 2% agarose gel. Primers were designed to target intron-spanning transcript regions when possible, and to amplify regions between 70-120 bp (Table 1.1 and 1.2). Primers for each of the target genes were designed using Primer3 software (Rozen and Skaletsky 2000) as implemented in the Geneious software version 11.0.2 (http://www.geneious.com, Kearse *et al.* 2012). All primers used in a single BioMark® (Fluidigm, San Francisco, CA) chip were evaluated for cross-amplification of targets using BLAST against the sequences of the full set of P450s. Primers were then evaluated with PCR and in a 2% agarose gel and re-designed if necessary.

High-throughput qPCR

Two 96-well plates were prepared per run, the first containing 29 cDNA samples consisting of the cDNA synthesized from the RNA samples, a no-template control, and pooled cDNA at serially diluted concentrations of 1, 1:10, 1:100, 1:1000, and 1:10000 made up from a mixture of all the samples, as well as the 200 µM forward and reverse mixtures of primers for each of the CYP3 clan P450s and housekeeping controls (Actin, EF alpha, GADPH, Rpl32, Tubulin). The second 96-well plate contained the same 29 cDNA samples, the 200 µM forward and reverse mixtures of primers for each of the CYP4 clan P450s, and housekeeping controls (Actin, EF alpha, GADPH, Rpl32, Tubulin). These plates were submitted to the Functional Genomics Unit of the William Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign where a microfluidics-based qPCR was run on a Biomark® 48x48 Fluidigm-Chip after a pre-amplification step of 15 cycles.

Quantification of cuticular hydrocarbons in eggs and adults

CHCs were extracted from 10 clusters of 30 eggs and 10 individual adults three to five days post-eclosion (based on preliminary experiments, showing that many of the identifiable cuticular hydrocarbons were present at this age) from each strain, following methods by Nelson and Buckner (1995) with modifications. CHCs were extracted by submerging individual adults and or eggs for 10 minutes in 200 µl hexane (Sigma-Aldrich, St. Louis, MO); containing 1-bromooctadecane (Sigma-Aldrich, St. Louis, MO) as the internal standard; 25 ng per µl. Extracts were then transferred to clean glass vials. The adults and or eggs were rinsed with an additional 200 µl of hexane containing the internal standard. Washed adults were inspected to ensure that

no damage had occurred. Extracts from the rinses were added to the initial extracts. Samples were stored at -4 ^{o}C until use.

Gas Chromatography-Mass Spectrophotometry (GC-MS) Analysis of Cuticular Hydrocarbons

Gas chromatography-mass spectrophotometry (GC-MS) analysis of extracted cuticular hydrocarbons was done on a Hewlett-Packard (HP) 6890 GC (Hewlett-Packard, Sunnyvale, CA, USA) in splitless mode, interfaced to an HP 5973 mass selective detector (MSD), with helium carrier gas. The column was programmed from 100^{o}C/2 min, 50^{o}C/min to 250^{o}C, then 250 to 320^{o}C at 4^{o}C/min. Injector and transfer line temperatures were 320^{o}C. Prior to GC-MS analysis, samples were removed from the refrigerator. They were concentrated to dryness under a steady stream of nitrogen, and then resuspended in 30 µl of hexane with standard from which 1 µl was injected into GC-MS for analysis. A control sample of hexane was run through the GC-MS every day before samples were analyzed to confirm that the GC column was clean. Each adult and egg cluster was analyzed as individual replicates from each strain. Hydrocarbon peaks were identified based on the relative retention time (J. Millar, University of California, Riverside). The abundance of each identified hydrocarbon peak was calculated relative to the internal standard.

Neonate feeding assays

I used a semi-synthetic artificial diet (Waldbauer 1984) for feeding assays to establish bifenthrin median-lethal concentrations (LC_{50}) with first instar larvae for the ALM and R347 strains. In feeding assays with neonates, bifenthrin was stirred into the diet at different concentrations (ALM – 2 ppm, 5 ppm, 10 ppm, 12 ppm, 15 ppm, 24 ppm; R347 – 8 ppm, 16

ppm, 24 ppm, 48 ppm, 75 ppm) and poured into separate 28 g cups to set (Niu *et al.* 2012). Four neonates were transferred with a soft brush into each plastic cup containing diets with bifenthrin or methanol as the solvent control for each strain. Twenty larvae from each strain were exposed to their respective bifenthrin concentrations, and each group of 20 larvae was replicated three times per concentration. Neonate mortality on diets was assessed after 48 h and scored according to a movement response when touched by a soft brush.

Egg mortality and contact toxicity assays

Eggs were selected within 24 h after turning pink – an indication that fertilization had occurred (Wade 1961). Eggs oviposited on paper towels were counted under microscope, after which the oviposition paper was cut into strips containing 25 fertilized eggs per strip. Strips were cut so that eggs were evenly dispersed within strips and not distributed in clusters greater than four in order to mitigate potential effects of larval cannibalism at high density (Bush *et al.* 2017). The eggs strips were then pinned (Bioquip, black enameled - size 0) to the center of filter papers (Whatman 1004-090 Grade 4 Qualitative Filter Paper). Each strip of eggs pinned to a single filter paper was then sprayed with a 1.5 mL solution containing 1 mL water and 0.5 mL bifenthrin (in methanol) using a spray gun kit (Badger, Franklin Park, IL) at the following concentrations: 5 ppm, 10 ppm, 20 ppm, and 40 ppm. Sprayed eggs pinned to filter papers were then placed inside Petri dishes (100 x 15 mm, Corning Incorporated, NY) on top of the same wheat bran diet used for rearing larvae. All sprays were repeated to include a total of ten egg strips and filter papers per concentration. Egg mortality and larval mortality were scored together for all unhatched eggs and larvae dead on the surface of filter paper after four days. Survivors of bifenthrin exposure that consumed bran diet were counted after three weeks. A larva was recorded as “normal” if it

reached the fourth or fifth instar based on typical development on rearing diet in the absence of insecticide. First through third instars were recorded as "stunted" (by exposure to the methanol solvent and solvent + insecticide) after the three weeks. Any replicates which produced egg mortality greater than two standard deviations from the mean at their respective concentrations were considered outliers and removed from further analysis.

qPCR Data analysis

Data collected from the Biomark® platform were assessed for quality with the Real-Time PCR Analysis software (Fluidigm) utilizing a quality threshold of 0.8 (quality scores ranging from 0 to 1, with 1 being an ideal exponential amplification curve). Melting curves were also evaluated for secondary peaks for each of the 2,304 assays. To correct for fluorescence drift and other background noise, a baseline correction for the amplification curve was set utilizing the linear-derivative method (Fluidigm Real-Time PCR Analysis, Fluidigm). Ct-values were obtained by setting a qPCR cycle threshold in a by-gene basis (i.e., setting the "by-detector" option in the Fluidigm software) to account for variability between primer pairs and to allow for assays of each gene to be treated as a separate experiment. I then used SAS (SAS University Edition v. 9.4, SAS Institute Cory, NC) to process and analyze the data starting from the obtained Ct values. The delta-Ct value (ΔCt) for each of the assays was calculated by subtracting the Ct-value for each reaction from that of the chosen reference housekeeping gene GADPH in the same sample, and this value was used to calculate statistics. One-way and multi-factor analysis of variance (ANOVA) were carried out after checking assumptions of independence by analyzing the distribution of residuals for normality, scatterplots of predicted vs. residual values for independence of variances, and a Bartlett's test for equal variances in the case of the

multifactor ANOVA. All pairwise tests were corrected for multi-testing with the false discovery rate (FDR) method. The negative of the estimate from the t-test is equivalent to the $\log_2$ scaled ΔΔCt, and this measure was used to report differential expression between pairs of treatments, strains, and combination of both factors.

Bioassay and CHC data analysis

Probit analysis (SPSS version 24, SPSS Inc., Chicago, IL) was used to determine the median-lethal concentrations (LC_{50}) following 48 hours of bifenthrin exposure in feeding assays with artificial diets. A two-way ANOVA (SPSS version 24, SPSS Inc., Chicago, IL) was applied to identify differences in egg mortality, larval mortality, and total mortality in spray assays with bifenthrin. Total CHC counts across 10 egg clusters and 10 adults from each population were pooled and tested for significance using the Student's t-test. Differences were considered significant if the 95% confidence intervals did not overlap with each other from the Probit analysis and if $P < 0.05$ for the Student's t-test.

RESULTS

Analysis of control and bifenthrin effects

I report differences in P450 expression of controls as indicators of constitutive expression (ALM control vs R347 control, R347 control vs ALM control) (Table 1.3), and upregulation or downregulation from bifenthrin in both strains (ALM control vs ALM bifenthrin, R347 control vs R347 bifenthrin) (Table 1.4). Because of the stringency of the FDR correction factor, I chose to report all expression differences as significant when $P < 0.1$. Only P450s with ≥ 2 fold-change were reported. Six P450s were constitutively overexpressed in the CYP3 clan, four of which

occurred in the ALM strain and two in R347 (Table 1.3). A total of 10 P450s in the CYP4 clan were constitutively overexpressed, seven of which occurred in R347 and three in the ALM strain (Table 1.3). Of the seven P450s in R347 constitutively overexpressed in the CYP4 clan, six of them occurred as pairs within the CYP4G, CYP341, and CYP367 subfamilies. The two P450s with the highest difference in expression between strains occurred in R347 with the CYP4 clan P450s CYP4G89 and CYP340AJ1 at 33.04 and 26.77 fold-change, respectively. The only two P450s that displayed increased expression with bifenthrin occurred in the ALM strain with CYP321C1v2 in the CYP3 clan and CYP367B8 in the CYP4 clan (Table 1.4). Bifenthrin treatment resulted in downregulation of multiple P450s in the CYP4 clan of each strain relative to their respective controls. In the ALM strain, P450s downregulated included CYP4AU1, CYP4AU2, and CYP4AU8. In R347, CYP4G89 and CYP340AJ1 were severely downregulated by the bifenthrin treatment, with fold-changes of -39.81 and -45.77, respectively.

CYP3 and CYP4 P450 exclusions

I disregarded results from P450s that did not meet the quality detection threshold of 0.8 in the Fluidigm analysis software across each sample. These included CYP6B44v2, CYP6B54, CYP6B55, CYP6B56, and CYP6AE55 for the CYP3 clan and CYP341J2 and CYP341M3 for the CYP4 clan. Additionally, amplification results were highly variable in the ALM strain controls for CYP341K1, CYP341J1, and CYP341M-, which affected comparisons with R347 and produced constitutive overexpression differences in these P450s at 84.7, 272.2, and 281.2-fold, respectively. Although I tested primer specificity, these P450s fall into groups of very closely related sequences. Due to amplification inconsistency in the ALM controls, I decided to

remove these from significant results and attribute the failures of these P450s in part to primer specificity.

Hydrocarbons

Identities of CHCs detected and quantities in eggs and adults from each strain are listed in Tables 1.5, 1.6, and 1.7. Total CHCs extracted were greater in the resistant strain for pooled samples of eggs (t = 3.40, df = 18, P = 0.003) and adults (t = 5.21, df = 18, P < 0.001). There were 79.3% more total CHCs extracted from R347 eggs than from the susceptible ALM strain eggs (Table 1.5). There were 114.94% more CHCs extracted from R347 adults than susceptible ALM adults (Tables 1.6-1.7).

Feeding assays with bifenthrin

Mortality data for bifenthrin in feeding assays fit the Probit model as measured by the goodness-of-fit test (P > 0.05). The LC_{50} after 48 hours of exposure to bifenthrin diet was 7.4 (5.9 – 9.6, 95% CI) for the ALM strain and 24.3 (18.2 – 33.1) for R347 (Table 1.8). The R347 LC_{50} was considered significantly greater because the 95% confidence interval did not overlap with the confidence interval of the ALM strain from the Probit analysis. The resistance factor, calculated as the ratio of the R347 LC_{50} to the ALM LC_{50}, was 3.3 (1.9 – 5.6).

Egg and contact toxicity assays

There was no significant interaction between strain and bifenthrin concentration for each of the two-way ANOVAs examining egg mortality and larval mortality. However, main effects of strain and bifenthrin concentration were observed for egg mortality (strain: F (1,99) = 6.411, P

= 0.013; concentration: F (4,99) = 12.827, $P < 0.001$) (Table 5). Pairwise comparisons using Least Squares Means revealed significant reductions in percent egg mortality at 5 ppm bifenthrin ($P = 0.028$) and at 10 ppm in R347 ($P = 0.008$) (Table 1.9). There were no differences in larval mortality between strains from bifenthrin exposure on filter papers.

Larval survivorship three weeks after bifenthrin exposure

Separate two-way ANOVAs were conducted using the number of surviving larvae considered as normal and another using the total number recorded as both normal and stunted for each strain (Table 1.10). In the analysis of normal larvae, there were no differences in survivorship between strains. However, when normal larvae and stunted larvae were pooled for each strain and tested for total survivorship, there were significant main effects of strain (F (1,99) = 7.722, $P = 0.007$) and bifenthrin concentration (F (4,99) = 51.525, $P < 0.001$) but no interaction between them. Pairwise comparisons using Least Squares Means revealed increases in total larval survivorship in R347 at 20 ppm bifenthrin ($P = 0.02$) and at 40 ppm ($P = 0.054$), which is borderline significant.

DISCUSSION

Constitutive overexpression of P450s is a mechanism contributing to pyrethroid resistance in many insect pests across multiple orders (Yang *et al.* 2006, Yang and Liu 2011, Liu *et al.* 2011, Zhen *et al.* 2018). In this study, the majority of expression differences between the resistant and susceptible *A. transitella* strains unexpectedly occurred in comparisons of controls, suggesting constitutive overexpression as a mechanism for pyrethoid resistance in *A. transitella*. In total, nine P450s displayed elevated expression in R347 controls versus four in ALM. Of the

nine P450s overexpressed in resistant controls, seven of them were in the CYP4 clan. Discerning functions of P450s, particularly in the CYP4 clan, remains a challenge as research on xenobiotic metabolism of specific subfamilies is limited. In *Plutella xylostella*, Yu *et al.* (2015) measured stage-specific P450 expression and found that CYP340s were expressed in fourth instar midguts and adult heads and suggested potential roles in xenobiotic metabolism as larvae and olfaction as adults for these families. Gao *et al.* (2016) found CYP340AW1 was inducible by abamectin in *P. xylostella* and more highly expressed in resistant larvae, further supporting the metabolism of xenobiotics as a function for P450s in the CYP340 family. CYP340AJ1 was constitutively overexpressed in resistant larvae that fed on control diets, but the extent of xenobiotic metabolism in *A. transitella* CYP340 enzymes is unknown.

Two of the CYP4 clan P450s constitutively overexpressed in the resistant strain were in the CYP4G subfamily linked to insecticide resistance via cuticular modifications. Detection of overexpression of multiple CYP4Gs in the pyrethroid-resistant strain suggests the possibility that resistant *A. transitella* may produce more cuticular hydrocarbons as a resistance mechanism. The elevated expression of CYP4Gs in the resistant strain was supported by detection of increased CHCs in eggs and adults from the resistant strain. More than 20 years ago, elevated constitutive expression of CYP4Gs in a lepidopteran agricultural pest, *Helicoverpa armigera*, was linked to pyrethroid resistance (Pittendrigh *et al.* 1997), although no mechanistic explanation was provided.

Expression of multiple CYP4Gs in the midgut was unexpected, in view of the fact that CHC-synthesis has been previously associated with oenocytes in resistant pests (Balabanidou *et al.* 2016). In *D. melanogaster* and *M. domestica*, CYP4G1 and CYP4G2 both encode oxidative decarbonylases as precursors to CHC synthesis, but only CYP4G1 expression was identified in

the oenocytes (Qiu *et al.* 2012). CYP4G15 in *D. melanogaster* is expressed in the nervous system (Maïbèche-Coisne *et al.* 2000), which indicates that CYP4G expression may not be restricted to oenocytes in other insects. In an RNA-Seq experiment with *A. transitella* larvae, consumption of diets containing the phytochemical furanocoumarin was associated with CYP4G89 expression in the midgut, although at lower levels than many CYP3 Clan P450s (Noble 2013, Calla *et al.* 2017). Balabanidou *et al.* (2016) and Yahouédo *et al.* (2017) measured epicuticle thickness with transmission electron microscopy in order to confirm differences in pyrethroid-resistant mosquitoes and a similar approach would help to illuminate the precise relationship between CYP4Gs, CHCs, and bifenthrin resistance in *A. transitella*.

In the susceptible ALM strain, CYP321C1v2 is a candidate likely involved in bifenthrin metabolism. Such a function is consistent with catalytic activities of CYP321 enzymes in other polyphagous lepidopterans. In the highly polyphagous corn earworm (*Heliocoverpa zea*), CYP321A1 metabolizes a broad range of phytochemicals (xanthotoxin, angelicin, anaphthoflavone), as well as insecticides, including the pyrethroid cypermethrin, diazinon, and aldrin (Li *et al.* 2004; Sasabe *et al.* 2004, Rupasinghe *et al.* 2007). CYP321A1 in *H. zea* owes its broad substrate specificity to a more spacious cavity that enables larger molecules to access the catalytic site (Rupasinghe *et al.* 2007). In the polyphagous *Spodoptera litura*, CYP321B1 has been implicated in the metabolism of the organophosphate chlorpyrifos and of the pyrethroid beta-cypermethrin (Wang *et al.* 2017).

The majority of P450s detected in this study were constitutively highly expressed in the controls of both strains, but P450s in both strains were also downregulated in response to bifenthrin selection. Little is known about the mechanisms associated with P450 downregulation, but some hypotheses include it as a homeostatic response to toxins or a need to divert

transcriptional machinery and energy for synthesis of components involved in detoxification (Davies *et al.* 2006, Yang and Liu 2011). Differential regulation of P450s in response to insecticides has been observed in the cotton bollworm *Heliocoverpa armigera*, the southern house mosquito *Culex quinquefasciatus*, and the Oriental fruit fly *Bactrocera dorsalis* (Zhou *et al.* 2010, Yang and Liu 2011, Huang *et al.* 2013). It is possible that the downregulation of P450s observed in the ALM and R347 strains may result from a need to increase production of other enzymes involved in the detoxification of bifenthrin.

In almonds, insecticides are applied during hull split after the suture widens and exposes the shell/kernel in order to suppress adult populations and deposit residues that either kill eggs directly or prevent neonates from penetrating the kernel (Siegel *et al.* 2019). Hatching neonates are exposed to insecticides as they wander across treated hulls and shells before they tunnel into kernels. The potential for modifications in the *A. transitella* cuticle that reduce insecticide penetration in the R347 strain are supported in part by multiple bioassays designed to mimic bifenthrin field exposure through direct spray and across treated surfaces. In assays with bifenthrin mixed into artificial diets, toxin exposure occurs through a combination of ingestion and contact exposure on the surface of the diet. A second set of bioassays showed that resistant eggs may have modifications that reduce insecticide penetration through the chorion at low concentrations. Whether or not CHCs contribute to reductions in egg mortality is an open question. The bifenthrin spray assays were ineffective at detecting neonate mortality, but the three-week larval assessment revealed that the combination of hatching from the bifenthrin-coated egg and wandering across the treated filter paper reduces survivorship as bifenthrin concentrations increase. The relative importance of CHCs in this tolerance, however, has not been definitively determined.

Comparative analyses of eggs are frequently underrepresented in resistance studies relative to host-damaging life stages. Although insecticide resistance has been documented in eggs of insect pests (Rodriguez *et al.* 2011), the mechanism(s) contributing to enhanced survival of resistant eggs are largely unknown. Differences in egg susceptibility of insect species to insecticides may be due to variations in the chorion that facilitate the uptake of oxygen and reduce penetration of insecticides; however, susceptibility to insecticides is also influenced by age and changes during embryonic development (Campbell *et al.* 2016). The focus of my studies was on recently fertilized *A. transitella* eggs, but eggs at other stages of development may differ in bifenthrin resistance.

Results from this study demonstrate the efficacy of applying whole transcriptome screening as an appropriate method for identifying candidate genes associated with insecticide metabolism and resistance. However, I acknowledge that the P450s represent a single enzyme superfamily. Glutathione-S-transferases (GSTs), carboxylesterases (COEs), and ABC transporters (ABCs) have all been linked pyrethroid resistance in other pest species (Achaleke *et al.* 2009, Labbé *et al.* 2013, Carvalho *et al.* 2013). and may represent additional avenues for exploration with *A. transitella*. Our analysis of cytochrome P450 expression in the CYP3 and CYP4 clans of *A. transitella* suggests multiple mechanisms may be contributing to bifenthrin resistance in populations collected from Kern County. Detection of elevated CYP4G expression in the resistant strain and subsequent confirmation of increased cuticular hydrocarbons in resistant populations indicates the cuticle may play a role in resistance acquisition in some capacity. Conventional management strategy for *A. transitella* suggests that insecticide applications in almonds during hull-split rotate based on mode of action; however, this approach disregards potential overlapping mechanisms of detoxification in *A. transitella* and may be

especially problematic if cuticular and/or chorion resistance enables cross-resistance to a diversity of synthetic organic insecticides representing different modes of action and different structural classes (Niu *et al.* 2012, Demkovich *et al.* 2015b). The potential crop losses due to resistance should concern tree nut growers and warrant the development of new strategies which disrupt these mechanisms.

TABLES

Table 1.1. Primer sequences for all CYP3 clan P450s used in qRT-PCR experiments.

P450	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon size (bp)
CYP6AB11v2	GAGAGAAAGCGAAATGTGGTC	TCACTTCACACAAATTGCATTTTCT	99
CYP6AB39	AAGATGGCCTAAAATAGACCCAAA	TTCTCTCGTTAAACATCAACGGC	110
CYP6AB40	CCAAGAGGCACAAGTTCGTG	AACCTGCCACTGACTGCATT	93
CYP6AB41	GTTTGGAGAGGGACCCAGAG	TCTGTAGGACAGCTGCTAGTC	83
CYP6AB42	CATAGGACCACGCGCTTGTA	CTGGCTTCACGGTGAACTTG	95
CYP6AB43	GGTGAAGGTCCTCGTTCATGT	GCGAGATAGTACTGCAGCCA	82
CYP6AB44	ATTTGGAATCGGACCACGCA	TTGACAATATGGCCGCCAAG	83
CYP6AB110v1	AGAGATGACTTACTTGGAGTGTGT	CACACTCCCGCATCAGGTAG	80
CYP6AE53	TGGATGTCATAGGATCATGTGCA	AAACGTTTCTGAGAATATCAAACAGC	110
CYP6AE54	AGAATCAAGAGGCTCAGGCA	TCATCAACACAGGCTTCCGT	121
CYP6AE55	ATGTGCATGGAGTAAGGCAT	AGAGTAGCTCTCCGATGTTTGT	135
CYP6AE56	CGGTTTGTGTGCATTTGGC	TGTATGACTCCATAAACATTATAGCTG	101
CYP6AE57	CTCTGCCTTGTGGTGCAGTA	GGTTCGGGGAAGAAGTCTGG	91
CYP6AE58	GCAGCAACAATACGTCGGAC	TGGATGCTGTACCATCAGGG	112
CYP6ML1	ATCGCTTCAAGATCGTCATCA	AGAAGGTCCCCGGAACTGTA	80
CYP6AN17	AACCGGCAGCCTTTTACGTA	ATCCCGCCAAAGACTGCATT	91
CYP6AW1	GCCCCGTCAGTCAGAGTATC	TGTGTACTATATCATGGTGCGGT	95
CYP6B44v2	ATACCTTTTGGGGTCGGACC	GCAGCTTCATCATGCACACT	82
CYP6B54	TTGTACAACAGAGCTTTCATATGAA	CACATCTTTATTCTCGACGCTCG	109
CYP6B55	TCCCTACCGAGTCGTTACCA	ACCATACTGAATAAACCGCGGT	189
CYP6B56	TGTGCGCATATACCCTTTGGA	GCTAAACAAATGCGACTCTGC	83
CYP6CV3	TCTGCCTTTTGGAGACGGAC	GAAATGCGGCCATTCCCATC	83
CYP9A63	ACACGGAATTTCGAGAGGGTC	CCAGCTCCACGTCTCTGATC	100
CYP9A64	GCAAGCGGATCCTTTCTTCG	TGAACGCTGGACTCAAGGTC	89
CYP9A65	TGCTCCCGTTCATGACAGAG	GATGACGTCATTGGCGTAGC	131
CYP9A66	CCAGATGGTGATGACCCTCA	CGCGATGACGTCATTTGTACA	106
CYP9G13	ATTGTGACGTTCGTGGTTGC	ATAGGCACCCCGGGTACATA	98
CYP9G30	ATGTCTATGGTGCTAGCAATCCT	TCCACGACATCCTCTGGGAA	80
CYP321C1v2	ACGGACAAGCCAGTGTTTCA	GAAGTTTCCAAAGCCGACGG	81
CYP321C3	GCATAAGACTGCTGCCTCCT	TGCCACAAATACTTTAGTCCCC	110
CYP321C4	GCTGTGTTTGGAGTGGAATCAG	AACGTAGATTTGAAAGCTCGTGT	80
CYP321C12v1	ACTCTTTTTACCACATGCCCG	CCGGGTCTAAAGCCAGGAAG	94
CYP321C12v2	GGCTTCATGACTCCAACGCT	GATCTCGGTCCCTCGTTGAC	99
CYP321F6	ACGTTTTTCACACCGTCCCT	ATCTCGAACCCTCTCTCGCT	98
CYP324A11	TGGGGAAGTCTCACGTTTCTG	GCCGCCAGAACAACCAGATA	106
CYP324A27	CGAAGCGATGTTAAAGCCGG	GCTGTCTACCCCAAAAGAGG	99
CYP18A1	AGAGGAATTCACTGGACGGC	TGTAAGAAGCGTCGCTGGTT	108
CYP333A1	GAGATGCTGCTCGCTGGTAT	TCGCGAAGTTTCTCCTGCTT	98
CYP354A11	TTCAAAGGAAACCAATTCGGAGG	GCTTTTATGAGCTCTGGGTCC	80
CYP365A1	TACCGCAGACGCCATAGTTC	AGAGCCACTGCAAAGGGATC	84

Table 1.2. Primer sequences for all CYP4 clan P450s used in qRT-PCR experiments.

P450	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon size (bp)
CYP4AU1	CCGACCGCTTTCTTCCTGAA	CAGCATGCCGAAATGACGTC	104
CYP4AU2	CACTGCGTGCACTCTGGATA	CAGGTATGGCGTATCGGGTG	85
CYP4AU8	TACCTGGGTGTGATTGGGGA	CATCCATCCGCTCACCCATT	148
CYP4G88	ACACAACTCCTTGACATCATCCA	TGGTTTCACTTAGGACGGAAGG	103
CYP4G89	TCTGGCACTAGGGAAAACGC	TTCCAAACCAAATGCGAGCG	92
CYP4G170	TTTTCAAACCTTGGCTGGGC	CGTTGGCGCTATTAATTTCCGA	83
CYP4L26	TCGAACCCCAACTTTGCAGA	AGTCCATAACCAAGCCAGGG	98
CYP340AJ1	GCCGCCCTAGAAGCTGTATT	CAACGAGCGGTCCAAACAAG	96
CYP340Q10	TACCTGATAACCCCAACGCG	TCATTGACATGTAGGCGTAGGA	84
CYP341A26	GTTTGTGGCCCTTTGTCAGC	TTGGGGTTCGATTGAGCGTT	88
CYP341J1	CCTGATCAGCGCGTGGAT	GCACACCTTCATCACCACCT	82
CYP341J2	TGGGATCGTGAGCATATGGT	ACTCCCTGATCCGACGAGAT	139
CYP341J3	AGGTCACGGGATACTCTATGGA	AGGATCGGGGCACTGGAT	96
CYP341K1	TTTTTGGCGTTCAGTTGCGG	GCAGGATATCGCAGTTTTGGT	81
CYP341L1	ACTTTATCCGCCTGTGCCAG	CGTCGGGCACAAGTTTGAAA	83
CYP341M2	TAGACTTTTCACCCGCACGA	TGAGACAGCAGAGCAGACAC	87
CYP341M3	CGGAAGGTGGGGTCTAAACA	CCGTTAGTGATAGCGTGGTCA	119
CYP341S1	CCTTTCAGTAACGGTGCAAGG	CGTCTGATGATTTGTGCCATGA	86
CYP341S2	CTGAGATAATACAATCAAAAAGACTCA	AGGTTTAGATATTTCTTTTTCACA	98
CYP341T1	CTCGAGGGCCCGTTGAAA	AGCAAACTGAGACCCAACACA	81
CYP367A1	CAGTCCAACATTTCACGGCG	CGTGACACTTGGATCTTTGCTC	83
CYP367B8	TGATCCTGACTGACCCCGAT	CCCCGAGCACCTCATACATA	99
CYP4M35	GTTTGAGGGACACGATACGACT	TGTCCTGTTTGTCCCTGTTGTT	80
CYP4M36	CGCCATCCTTATTCATACATACCG	CTGACTTCATTTCCATCATTGCG	85
CYP4M37	ATTAGACCGTGGTTGAAAGAAGG	GCTGGTGTTAAAATCTTTCTTCGC	80
CYP341M1	GGCAGTGTTCCAGAGTTATCCT	GTGGACATCATGGAGAACTGGA	86
CYP341M--	GCTTACACTGTGGACATAACTGG	TGATACCGTTGATGGCCAGT	100

Table 1.3. Fold-change of P450s constitutively overexpressed in the midguts of larvae that fed on control (methanol) artificial diets in the susceptible strain (ALM) and pyrethroid-resistant strain (R347) of *A. transitella*.

Constitutively Overexpressed in R347	Fold-change	*t* -statistic	DF	*P* -value
CYP3 clan				
CYP6AE54	11.72	6.03	19	<0.01
CYP6AN17	8.83	3.90	20	0.046
CYP4 clan				
CYP4G89	33.04	2.78	20	0.054
CYP4G170	5.13	3.63	20	0.028
CYP340AJ1	26.77	2.69	20	0.055
CYP341S1	4.08	3.49	20	0.035
CYP341T1	18.72	2.88	20	0.048
CYP367B8	3.25	4.20	20	0.015
CYP367A1	2.78	2.73	20	0.053
Constitutively Overexpressed in ALM				
CYP3 clan				
CYP6AB40	20.92	4.07	20	0.037
CYP4 clan				
CYP4AU1	2.47	3.02	20	0.042
CYP4AU2	2.01	3.42	20	0.033
CYP4AU8	2.12	3.76	20	0.028

Table 1.4. Fold-change of P450s upregulated and downregulated in the midguts of larvae that fed on artificial diets containing 0.5 ppm bifenthrin in the susceptible strain (ALM) and pyrethroid-resistant strain (R347) of *A. transitella*.

Upregulated by Bifenthrin in ALM	Fold-change	*t* -statistic	DF	*P* -value
CYP3 clan				
CYP321C1v2	5.12	3.14	20	0.077
CYP4 clan				
CYP367B8	2.00	2.46	20	0.081
Downregulated by Bifenthrin in ALM				
CYP4 clan				
CYP4AU1	-3.13	3.81	20	0.030
CYP4AU2	-2.40	4.28	20	0.017
CYP4AU8	-2.47	4.51	20	0.015
Downregulated by Bifenthrin in R347				
CYP4 clan				
CYP4G89	-39.81	2.93	20	0.045
CYP340AJ1	-45.77	3.13	20	0.040

Table 1.5. Cuticular hydrocarbons (CHCs) extracted from 10 clusters of 30 eggs (R1-R10) in the susceptible ALM and pyrethroid-resistant (R347) populations and identified through GC-MS analysis.

ALM Population

	CHC / Egg										
CHC Identity	RI	R2	R3	R4	R5	R6	R7	R8	R9	RI0	Mean (± SE)
Octadecane + octadecane alkene	38.71	39.85	41.28	45.52	29.71	49.49	49.85	41.28	35.30	37.73	40.87 ± 1.97
Tricosane	109.12	57.34	56.48	59.42	181.75	44.61	38.35	56.48	171.02	215.79	99.04 ± 20.90
Tetracosane	50.91	35.33	26.89	32.61	59.64	27.91	28.10	26.89	73.72	76.19	43.82 ± 6.25
Pentacosane	207.56	175.82	198.39	179.37	296.83	117.97	114.71	198.39	470.07	377.63	233.67 ± 36.15
Hexacosane	17.80	24.59	14.57	17.15	22.28	14.32	15.74	14.57	36.89	37.01	21.49 ± 2.79
Heptacosane	156.54	115.92	149.35	116.46	216.89	75.20	70.00	149.35	227.92	226.80	150.44 ± 18.49
Octacosane	18.96	19.65	16.17	14.50	23.76	0.00	12.71	16.17	38.93	40.17	20.10 ± 3.79
Nonacosane	186.00	112.72	167.80	118.55	281.07	75.39	57.01	167.80	323.57	233.70	172.36 ± 27.45
Hentriacontane	16.04	15.12	13.58	11.59	28.67	36.48	21.87	13.58	48.01	50.19	25.51 ± 4.64
TOTAL	**801.63**	**596.34**	**684.51**	**595.18**	**1140.61**	**441.36**	**408.36**	**684.51**	**1425.43**	**1295.23**	**807.32 ± 112.74**

R347 Population

	CHC / Egg										
CHC Identity	RI	R2	R3	R4	R5	R6	R7	R8	R9	RI0	Mean (± SE)
Octadecane + octadecane alkene	52.14	39.93	47.61	42.55	55.31	30.57	50.84	39.92	34.41	50.63	44.39 ± 2.59

Table 1.5 (continued)

Tricosane	193.70	192.57	49.86	48.35	204.49	193.28	295.13	101.52	150.52	64.98	149.44 ± 25.73
Tetracosane	123.83	114.30	35.53	36.63	117.88	81.63	117.44	37.56	62.57	39.52	76.69 ± 12.22
Pentacosane	528.46	566.16	267.96	327.73	546.97	504.57	518.84	541.08	737.18	294.30	483.33 ± 45.80
Hexacosane	59.26	65.61	19.01	24.36	52.70	23.06	51.22	28.48	43.02	20.64	38.73 ± 5.56
Heptacosane	330.94	360.99	148.95	189.15	328.44	274.02	315.98	310.55	423.08	152.20	283.43 ± 29.04
Octacosane	41.89	34.69	14.63	21.01	36.27	21.38	29.31	26.13	40.80	16.60	28.27 ± 3.12
Nonacosane	378.21	414.37	129.70	188.38	417.51	307.42	379.88	331.00	447.69	126.56	312.07 ± 38.34
Hentriacontane	39.59	46.30	14.15	17.61	43.18	26.75	44.06	28.18	38.47	15.53	31.38 ± 3.96
TOTAL	**1748.01**	**1834.92**	**727.39**	**895.77**	**1802.76**	**1462.68**	**1802.72**	**1444.41**	**1977.74**	**780.95**	**1447.73 ± 150.61**

Table 1.6. Cuticular hydrocarbons (CHCs) extracted from ten adult moths (R1 – R10) in the susceptible ALM population and identified through GC-MS analysis.

ALM Population											
	CHC / Adult										
CHC Identity	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	Mean (± SE)
Octadecane + octadecane alkene	47.24	48.00	44.06	41.96	43.43	47.14	53.85	48.95	46.66	32.76	45.40 ± 1.75
Tricosane	27.17	48.00	39.95	38.54	46.40	56.60	134.65	28.37	33.78	136.67	59.01 ± 13.09
Tetracosane	20.09	22.21	17.61	17.65	14.48	17.65	24.85	13.80	13.99	34.43	19.67 ± 1.99
Pentacosane	404.24	621.34	367.92	336.57	420.79	457.95	603.88	317.69	340.28	959.63	483.03 ± 62.72
11-Methyl + 13-methyl-pentacosane	36.83	80.45	33.49	40.01	62.21	71.02	60.18	53.61	31.52	115.66	58.50 ± 8.24
5-Methyl-pentacosane	8.53	21.54	9.38	11.85	15.91	18.83	14.45	15.34	9.80	37.16	16.28 ± 2.67
3-Methyl-pentacosane	11.45	21.59	8.14	9.19	14.13	16.77	11.97	14.00	12.74	34.98	15.50 ± 2.48
Hexacosane	23.13	48.85	25.41	21.18	29.97	25.07	39.40	20.18	15.01	75.61	32.38 ± 5.73
Heptacosane	439.96	753.61	498.11	395.16	534.08	461.77	648.99	300.80	332.04	1409.27	577.38 ± 102.12
11-Methyl + 13-methyl-heptacosane	42.81	90.45	30.81	31.39	65.23	58.05	62.00	51.25	42.24	126.51	60.07 ± 9.28
7-Methyl-heptacosane	33.46	50.52	18.04	16.96	48.97	42.96	50.92	28.53	56.82	73.01	42.02 ± 5.62
5-Methyl-nonacosane	29.46	59.36	21.28	19.82	47.86	31.94	41.23	30.77	31.94	99.15	41.28 ± 7.46

Table 1.6 (continued)

Octacosane	31.93	32.84	21.36	20.67	19.55	16.87	22.34	24.43	23.13	52.44	26.56 ± 3.29
Nonacosane alkene	23.33	17.68	33.20	0	37.18	0	0	50.30	0	0	16.17 ± 6.02
Nonacosane	653.58	642.19	442.09	440.89	445.95	359.67	408.08	317.49	492.65	888.19	509.08 ± 54.26
Triacontane	18.58	16.04	0	18.58	16.04	0	0	0	0	17.58	8.68 ± 2.91
Hentriacontane	168.99	179.76	140.86	114.65	116.85	83.74	114.44	73.39	120.73	229.70	134.31 ± 14.89
Dotriacontane	0	0	0	0	0	0	0	0	0	10.20	1.02 ± 1.02
Ttitriacontane	44.44	34.04	36.46	22.56	20.06	13.17	23.61	22.13	32.46	42.77	29.17 ± 3.28
Pentatriacontane	0	0	0	0	0	0	0	0	0	0	0
13-Methyl-pentatriacontane	104.75	108.27	46.05	49.45	48.95	36.63	36.90	49.29	70.28	100.05	65.06 ± 9.07
13, 23-Dimethyl-pentacontriane	474.49	635.53	306.03	351.71	304.84	303.95	249.69	307.70	365.79	625.90	392.56 ± 43.87
Mix of methyl branched CHCs	163.06	91.86	73.77	86.08	80.76	55.89	70.23	67.97	85.74	96.88	87.22 ± 9.27
Mix of methyl branched CHCs	938.72	746.82	508.44	556.74	432.99	401.98	435.14	403.30	412.07	675.17	551.14 ± 57.41
TOTAL	**3746.24**	**4370.97**	**2722.44**	**2641.60**	**2866.63**	**2577.63**	**3106.78**	**2239.27**	**2569.66**	**5873.71**	**3271.49 ± 351.73**

Table 1.7. Cuticular hydrocarbons (CHCs) extracted from ten adult moths (R1 – R10) in the pyrethroid-resistant (R347) population and identified through GC-MS analysis.

R347 Population

	CHC / Adult										
CHC Identity	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	Mean (± SE)
Octadecane + octadecane alkene	67.28	50.18	53.42	53.44	37.03	37.00	36.36	53.44	50.18	54.63	49.29 ± 3.12
Tricosane	183.08	139.69	65.95	55.00	277.96	180.18	104.10	55.00	39.69	31.82	113.25 ± 25.46
Tetracosane	65.09	27.51	27.81	53.12	61.00	54.33	34.68	23.12	27.51	25.32	39.95 ± 5.20
Pentacosane	1539.96	723.28	790.76	1051.20	1690.54	1545.57	920.05	551.20	723.28	643.34	1017.92 ± 133.23
11-Methyl + 13-methyl-pentacosane	95.95	54.90	76.64	46.41	150.86	74.82	83.95	41.41	54.90	57.06	73.69 ± 10.18
5-Methyl-pentacosane	32.66	11.90	23.54	28.89	42.37	23.35	29.02	10.89	11.90	12.11	22.66 ± 3.42
3-Methyl-pentacosane	45.60	22.28	32.70	46.86	55.43	35.15	26.32	16.86	22.28	14.96	31.84 ± 4.35
Hexacosane	114.51	36.19	48.69	134.49	113.08	120.34	79.28	34.49	36.19	38.03	75.53 ± 13.05
Heptacosane	1914.31	915.91	931.67	756.57	1842.32	2007.63	1448.84	756.57	915.91	779.94	1226.97 ± 164.41
11-Methyl + 13-methyl-heptacosane	160.34	100.37	131.67	167.41	214.28	114.52	111.61	67.41	100.37	81.09	124.91 ± 14.07
7-Methyl-heptacosane	114.38	69.20	86.67	86.90	128.82	84.43	66.57	46.90	69.20	37.77	79.09 ± 8.79
5-Methyl-nonacosane	157.79	70.48	99.15	155.67	149.46	99.88	90.99	55.67	70.48	36.99	98.66 ± 13.59

Table 1.7 (continued)

Octacosane	139.27	70.15	79.43	66.08	100.45	134.45	69.75	56.08	70.15	70.28	85.61 ± 9.27
Nonacosane alkene	61.32	0.00	41.93	88.86	0.00	42.19	0.00	68.86	0.00	0.00	30.52 ± 10.93
Nonacosane	2049.34	1475.30	1297.39	1268.23	1448.66	1932.04	1137.67	1084.23	1475.30	1278.77	1444.69 ± 100.53
Triacontane	52.71	22.44	22.31	42.44	40.97	47.33	27.07	22.44	27.07	20.48	32.53 ± 3.81
Hentriacontane	683.05	280.83	288.30	538.94	577.30	588.35	369.19	338.94	280.83	265.92	421.17 ± 50.04
Dotriacontane	23.30	0.00	0.00	0.00	19.68	19.88	0.00	0.00	0.00	0.00	6.29 ± 3.22
Ttitriacontane	297.66	108.17	123.72	250.04	268.09	249.96	113.95	150.04	108.17	94.07	176.39 ± 25.26
Pentatriacontane	18.84	0.00	0.00	0.00	13.61	0.00	0.00	0.00	0.00	0.00	2.95 ± 2.20
13 Methyl-Pentatriacontane	250.08	115.06	171.67	115.50	117.41	104.08	141.96	85.50	115.06	87.53	130.38 ± 15.48
13, 23 - Dimethyl Pentacontriane	886.53	694.13	715.93	807.07	585.30	588.19	976.64	506.07	694.13	457.56	691.15 ± 52.11
Mix of methyl branched CHCs	361.39	116.30	135.92	102.89	143.03	108.27	114.97	100.89	116.30	73.42	137.34 ± 25.62
Mix of methyl branched CHCs	1483.89	710.44	683.21	1008.54	911.94	1000.59	1044.91	728.54	710.44	905.01	918.75 ± 76.65
TOTAL	**10798.33**	**5814.73**	**5928.47**	**6924.56**	**8989.60**	**9192.54**	**7027.88**	**4854.56**	**5719.35**	**5066.12**	**7031.61 ± 630.04**

Table 1.8. Median-lethal concentrations (LC_{50}) with 95% confidence intervals of first instar larvae in the susceptible (ALM) strain and pyrethroid-resistant strain (R347) that fed on artificial diet containing bifenthrin at different concentrations. The resistance ratio was calculated by dividing the LC_{50} of R347 by the LC_{50} of ALM.

Population	48 h LC_{50} (95% CI)	Resistance Factor
Susceptible (ALMOND)	7.4 ppm (5.9 - 9.6)	
Resistant (R347)	24.3 ppm (18.2 - 33.1)	3.3 (1.9 - 5.6)

Table 1.9. Egg mortality and larval mortality following bifenthrin sprays on filter papers at 5 ppm, 10 ppm, 20 ppm, 40 ppm in the susceptible strain (ALM) and resistant strain (R347) of *A. transitella*. Control solution consisted of 33% methanol, which was used as the solvent in all sprays. One rep was removed from R347 in the control, at 10, and at 20 ppm because the egg mortality exceeded two standard deviations from the mean. Parentheses indicate the standard error.

	ALM			R347		
Concentration	*n*	% Egg Mortality	% Larval Mortality	*n*	% Egg Mortality	% Larval Mortality
Control	400	10.5 (±1.4)	0	375	10.4 (±1.6)	0
5 ppm	250	16.4 (±2.1)*	2.4 (±0.9)	250	8.8 (±1.4)*	2.0 (±0.9)
10 ppm	250	22.0 (±2.5)*	9.6 (±1.8)	225	10.7 (±2.1)*	11.6 (±2.2)
20 ppm	250	29.6 (±6.7)	21.2 (±3.4)	225	28.4 (±5.6)	16.0 (±4.8)
40 ppm	250	30.4 (±4.5)	35.2 (±3.0)	250	28.8 (±5.5)	29.2 (±5.8)

* Difference between ALM and R347 at respective concentrations of bifenthrin were significantly different ($P < 0.05$)

Table 1.10. Larval survivorship on bran diet following bifenthrin egg sprays on filter papers at 5 ppm, 10 ppm, 20 ppm, and 40 ppm in the susceptible strain (ALM) and resistant strain (R347) of *A. transitella*. Control solution consisted of 33% methanol, which was used as the solvent in all sprays. "Normal" larvae were fourth or fifth instars while "stunted" larvae were first through third instar at the end of the trial period. One rep was removed from R347 in the control, at 10, and at 20 ppm because the egg mortality exceeded two standard deviations from the mean. Parentheses indicate the standard error.

	ALM				R347			
Concentration	*n*	% Normal	% Stunted	Total Survivorship	*n*	Normal	Stunted	Total Survivorship
Control	400	68.8 (±3)	4.8 (±2.1)	73.5 (±3.7)	375	65.1 (±2.1)	9.3 (±2.6)	74.4 (±2.9)
5 ppm	250	61.6 (±4.3)	4.8 (±1.8)	66.4 (±4.8)	250	63.2 (±4.8)	8.4 (±3.3)	71.6 (±4.2)
10 ppm	250	32.4 (±5.2)	12.8 (±3.8)	45.2 (±3.1)	225	35.6 (±5.2)	12.9 (±3.0)	48.4 (±5.4)
20 ppm	250	18.4 (±5.6)	8.8 (±2.8)	27.2 (±6.1)*	225	28.4 (±6.1)	16.4 (±3.4)	44.9 (±7.2)*
40 ppm	250	11.2 (±2.4)	4 (±1.7)	15.2 (±2.5)**	250	20.4 (±3.6)	7.6 (±2.5)	28.0 (±4.6)**

* Difference between ALM and R347 was significant ($P < 0.05$)

** Difference between ALM and R347 considered borderline significant ($P = 0.054$)

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CHAPTER 2

ANNOTATION OF THE CARBOXYLESTERASES IN THE GENOME OF THE NAVEL ORANGEWORM (*AMYELOIS TRANSITELLA*)

INTRODUCTION

Carboxylesterases (COEs) are an enzyme superfamily capable of hydrolyzing a broad range of ester-containing compounds and are present in animals, plants, insects, and microbes (Oakeshott *et al.* 2005). Insect carboxylesterases perform a variety of functions, including developmental and neurological processes, pheromone degradation, and metabolism of xenobiotics (Yu *et al.* 2009, Kamita and Hammock 2010, Montella *et al.* 2012, Durand *et al.* 2010, Feng *et al.* 2018) and are classified into subfamilies based on sequence, substrate specificity, and function. These subfamilies include alpha-esterases, juvenile hormone esterases, integument esterases, beta-esterases, acetylcholinesterases, gliotactins, glutactins, neuroligins, and neurotactins (Yin *et al.* 2011, Lu *et al.* 2015, Feng *et al.* 2017, Wu *et al.* 2018). The classification system for COEs developed by Oakeshott *et al.* (2005, 2010) was based on a comprehensive phylogeny that placed these subfamilies into clades separated within three distinctive classes: intracellular catalytic, secreted catalytic, and neurodevelopmental. The intracellular catalytic class is associated with dietary detoxification functions and consists of the alpha-esterases exclusively. The secreted catalytic class is involved in hormone and pheromone degradation and consists of the juvenile hormone esterases, integument esterases, beta-esterases, and glutactins (Tsubota and Shiotsuki 2010). The neurodevelopmental class contains the acetycholinesterases, gliotactins, neuroligins, neurotactins, and uncharacterized esterases. Carboxylesterases in the neurodevelopmental class are generally considered noncatalytic and

perform cell-to-cell communication functions as adhesive proteins, except for the acetylcholinesterases, which are catalytic and metabolize the neurotransmitter acetylcholine (Marchot and Chatonnet 2012, Johnson and Moore 2013).

The Oakeshott *et al.* (2005, 2010) classification has been applied for several insects with fully annotated COEs including *Aedes aegypti*, *Anopheles gambiae*, *Anopheles sinensis*, *Apis mellifera*, *Bombyx mori*, *Culex pipiens quinquefasciatus*, *Drosophila melanogaster*, *Leptinotarsa decemlineata*, *Musca domestica*, *Nasonia vitripennis*, *Tribolium castaneum*, and *Trichoplusia ni* (Claudianos *et al.* 2006, Strode *et al.* 2008, Yu *et al.* 2009, Oakeshott *et al.* 2010, Yan *et al.* 2012, Lu *et al.* 2015, Feng *et al.* 2018, Wu *et al.* 2018). Among these insects, the lepidopterans *Bombyx mori* and *Trichoplusia ni* have the greatest number of COEs, reflective of significant radiations in the alpha-esterases relative to other species; 55 of 76 COEs in *B. mori* and 57 of 87 COEs in *T. ni* belong to this subfamily. Alpha-esterases are associated with metabolism of xenobiotics and have undergone considerable expansions in insects, suggesting roles in the detoxification of insecticides and acquisition of resistance (Feng *et al.* 2018). However, recent assessments across entire suites of COEs have revealed that insecticide metabolism and resistance are not restricted to the alpha-esterases. In *Leptinotarsa decemlineata*, specific alpha-esterases and integument esterases are inducible by fipronil and cyhalothrin, multiple subfamilies of COEs including alpha-esterases, integument esterases, beta-esterases, and acetylcholinesterases may contribute to pyrethroid resistance in *Musca domestica*, and *Anopheles sinensis* (Lu *et al.* 2015, Feng *et al.* 2018, Wu *et al.* 2018).

Carboxylesterases considered to be catalytic hydrolyze different compounds containing carboxylic esters into their respective alcohol and acid metabolites (Zhang *et al.* 2014). Carboxylesterases in the intracellular and secreted catalytic class are able to perform these

reactions on endogenous and exogenous substrates because of a catalytic triad involving a conserved serine – acid – histine (GxSxG – E/D – H) (Oakeshott *et al.* 2005, Montella *et al.* 2012, Zhang *et al.* 2014). The triad is a signature motif present in all COEs, although not all subfamilies are catalytically active. With the exception of the catalytic acetylcholinesterases, the neuroligins, gliotactins, and neurotactins in the neurodevelopmental class of COEs generally contain one or more substitutions in the triad residues, which may explain their loss of metabolic activity and association with noncatalytic roles such as cell-to-cell communication (Yu *et al.* 2009, Wu *et al.* 2018).

The navel orangeworm *Amyelois transitella* is the most serious economic pest of almonds and pistachios in California orchards. Although management practices for this agricultural pest have emphasized sanitation awareness, improving application efficacy, and, more recently, implementing mating disruption, growers continue to apply insecticides to reduce damage to acceptable levels (Higbee and Siegel 2009, Higbee *et al.* 2017, Siegel *et al.* 2019). The pyrethroid insecticides are the most heavily applied class of insecticides in tree nuts as measured by acres treated (Demkovich *et al.* 2015, CDPR 2016). Resistance to bifenthrin was first reported in Kern County almond orchards in 2013 (Demkovich *et al.* 2015). Examining individuals from this resistant population with bioassays involving pyrethroids and synergists suggested that both cytochrome P450s and carboxylesterases may be involved in resistance (Demkovich *et al.* 2015). Because all synthetic pyrethroids registered for use against navel orangeworm (bifenthrin, beta-cyfluthrin, zeta-cypermethrin, esfenvalerate, fenpropathrin, lambda-cyhalothrin, permethrin) contain a carboxylic ester and are likely substrates for carboxylesterases, activity involving this enzyme superfamily may contribute to pyrethroid resistance in Kern County populations.

In order to investigate COE involvement in *A. transitella* pyrethroid resistance and identify candidate genes, it is essential to annotate all members of the enzyme superfamily. In this Chapter, I annotated all of the carboxylesterases in the *A. transitella* genome and separated them by subfamily according to the Oakeshott *et al.* (2005, 2010) classification system. Additionally, I constructed a phylogeny for lepidopteran carboxylesterases using fully annotated sets from *A. transitella*, *Bombyx mori*, *Plutella xylostella*, and *Trichoplusia ni* and compared results across species.

MATERIALS AND METHODS

A. transitella carboxylesterase identification

COEs were located in the automatic NCBI annotations performed in the genome assembly (https://i5k.nal.usda.gov/available-genome-browsers, NCBI Accession no: GCF_001186105.1). COE identifications were performed using BLAST searches of the genome against sets of insect COES downloaded from NCBI protein and nucleotide databases, UniProtKB/TrEMBL, and UniProtKB/Swiss-Prot. Additionally, amino acid sequences of fully annotated COEs were obtained for *Bombyx mori* (Yu *et al.* 2009), *Drosophila melanogaster* (FlyBase), *Plutella xylostella* (You *et al.* 2013), and *Trichoplusia ni* (Fu *et al.* 2018) and were used as queries to search *A. transitella* genome with an e-value cut-off of 1E-5. Reciprocal best-hit BLAST searches for the set of putative COEs against the databases were also conducted. All putative COEs were then manually verified and annotated in the i5kNal WebApollo online interface.

Genomic distribution, gene structure, and conserved domains

Scaffold location, mRNA sequences, and coding sequences (CDS) were extracted from each COE annotated in Web Apollo. Structures of COEs were generated with GSDS 2.0 (Hu *et al.* 2015) using intron/exon sites obtained from mRNA and CDS sequences. The catalytic triad GxSxG – E/D – H was located for each gene in *A. transitella* through BLASTP searches against the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) with COE amino acid sequences as queries.

Phylogeny of lepidopteran COEs

Amino acid sequences of annotated COEs for *B. mori*, *P. xylostella*, and *T. ni* were obtained and checked against models described through their respective NCBI genome assemblies. I removed COE fragments, duplicates, and genes that did not match their respective species through NCBI BLASTP searches. This resulted in COE reductions for *T. ni* and *P. xylostella*. Filtered COEs for *B. mori*, *P. xylostella*, and *T. ni* were aligned with *A. transitella* annotated COEs using MUSCLE 3.7 in CIPRES Gateway. A phylogenetic tree reconstruction for *B. mori*, *P. xylostella*, *T. ni*, and *A. transitella* COEs was conducted using maximum likelihood with the RAxML-HPC v.8 on XSEDE in CIPRES (Stamatakis, 2014) using the Protein GAMMA model, LG amino acid substitution model with bootstrap resampling of 1,000 replicates, and *Daphnia magna* neurotactin as the outgroup. Tree images were rendered with FIGTREE v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

COE Nomenclature

A. transitella COEs were named based on the subfamily (AE: alpha-esterase; JHE: juvenile hormone esterase; IE: integument esterase; BE: beta-esterase; ACE: acetylcholinesterase; GLI: gliotactin; NLG: neuroligin; NRT: neurotactin; UN: uncharacterized carboxylesterase) with a number corresponding to the order in which they were annotated. Placement of *A. transitella* COEs in subfamilies was determined through phylogenetic relationships with fully characterized COEs in *B. mori* and *T. ni*. Although utilized in the phylogeny, the classification of *P. xylostella* COEs into subfamilies was not described by You *et al.* (2013). The classification of *A. transitella* COEs into subfamilies was supported additionally through relationships identified in the *A. transitella*-specific phylogeny.

RESULTS

Genomic distribution and structure of *A. transitella* COEs:

Carboxylesterases tended to cluster together, with 16 identified as pairs on individual scaffolds, and 20 carboxylesterases in clusters of 3 or more on individual scaffolds (Table 2.1). The two largest clusters contained 5 carboxylesterases across a 67.5 kb space in scaffold NW_013535526.1 and 4 carboxylesterases across an 11.5 kb space in scaffold NW_013535408.1. The number of introns was relatively conserved within the intracellular catalytic alpha-esterases and the subfamilies in the secreted catalytic class of COEs. The majority of the alpha-esterases had two or three introns (40/45) and were primarily phase 0 and phase 2 in each gene (Figure 2.1). Most of the introns in these alpha-esterases, in particular the majority of first introns, were phase 0 introns (33/40, inserted between two consecutive codons), followed by phase 2 (36/40, between the second and third nucleotides of a codon). Only two COEs contained

a single intron, and three contained four introns or more. All subfamilies in the secreted catalytic class contained 7 – 9 introns, with the exception of AtraJHE1, which had three (Figure 2.2). The greatest variation occurred in the neurodevelopmental class of COEs, with intron counts ranging as low as two in AtraACE1 to as high as 14 in AtraGLI (Figure 2.3). The neuroligins constitute the largest subfamily of COEs in the neurodevelopmental class, with highly variable gene structures containing 8 – 13 introns.

Identification and classification of *A. transitella* COEs:

A total of 64 putative COEs were identified in the *A. transitella* genome (Table 2.2). The total number of carboxylesterases in *A. transitella* was greater than the number in *P. xylostella* but fewer than in *B. mori* and *T. ni*. *A. transitella* has 45 alpha-esterases, more than *P. xylostella* but fewer than *B. mori* and *T. ni*. *A. transitella* has the same number of integument esterases, beta-esterases, and uncharacterized esterases as *B. mori* and *T. ni* but fewer juvenile hormone esterases than *B mori*, *T ni*, and *P. xylostella*. I identified 2 acetylcholinesterases, 1 gliotactin, 6 neuroligins, 2 neurotactins, and 1 uncharacterized esterase in *A. transitella*, constituting the more conserved neurodevelopmental class. The distribution of COEs among the neurodevelopmental class in *A. transitella* was the same as in *B. mori* and *T. ni* for all subfamilies, except for the uncharacterized carboxylesterases.

Phylogeny of lepidopteran COEs:

In the maximum-likelihood phylogeny (Figure 2.4) representing 276 lepidopteran COEs across four species, I recovered clades containing the juvenile hormone esterase, integument esterase, beta-esterase, acetylcholinesterase, gliotactin, neuroligin, neurotactin, and

uncharacterized subfamilies for *A. transitella*, *B. mori*, *P. xylostella*, and *T. ni*. Bootstrap support for these clades (≥ 70) was high, with the exception of the beta-esterases, which had bootstrap support of 58. I detected multiple clades containing the alpha-esterases for these four species; however, deeper relationships within this subfamily and ones that connect the different subfamilies were poorly supported. Therefore, I could not adopt the Oakeshott classification system representing COEs as clades A-N based on this phylogeny and accordingly report overall counts within the subfamilies instead.

Lepidopteran phylogeny – intracellular catalytic class (alpha-esterases)

Unique expansions of supported alpha-esterases (AE) within *B. mori* and *T. ni* contribute to the elevated counts of total COEs in these species relative to *A. transitella* and *P. xylostella*. Phylogenetic comparison between *A. transitella*, *B. mori*, *P. xylostella*, and *T. ni* alpha-esterases presents several 1:1:1:1 orthologs but no unique expansions in the alpha-esterases in *A. transitella*. I recovered two supported clades entirely of alpha-esterases containing at least one substitution in the GxSxG – E/D – H motif constituting the consensus triad Ser – Glu/Asp – His (Figure 2.5, purple clades) . In each of these noncatalytic clades, multiple 1:1:1:1 orthologous relationships occur among the four lepidopteran species.

Lepidoptera phylogeny – secreted catalytic class

Supported clades for the juvenile hormone esterases (JHE), beta-esterases (BE), and integument esterases (IE) were recovered (Figure 2.4, green clade, blue clade, yellow clade). *A. transitella* has only two JHEs compared to the four identified in *B. mori* and *T. ni* and the seven present in *P. xylostella*. The shared amino acid identities were high between *A. transitella* JHEs

at 70.3%, indicating a potential duplication event (Table 2.3). There was support for a clade linking the beta-esterases and integument esterases together as sister groups. I identified 1:1:1:1 orthologs for each of the two beta-esterases within each species. AtraBE1 shared 62.5 – 68.1% identity with BmBE2, Px017920, and TnCOE80, while AtraBE2 shared 67.1 – 70.5% identity with BmBE1, Px011941, and TnCOE26 (Table 2.4). However, overall bootstrap support at 58 for this subfamily clade was low. Although I recovered a clade containing the integument esterases, the relationships within it were not supported by the bootstrap values and orthologs were not identifiable (Table 2.5).

Lepidopteran phylogeny – neurodevelopmental class

All neurodevelopmental esterases were placed in their respective subfamilies with high support, except for the neurotactins (NRT) (Figure 2.4, orange clades), which split into two separate clades (AtraNRT2, Bmnrt1, and TnCOE83; AtraNRT1, Bmnrt2, and TnCOE12). *A. transitella* neuroligins (NLG) (Figure 2.4, red clade) shared 21.3 – 49.7% identity with each other and 52.1 – 94.2% identity with their respective 1:1:1:1 orthologs from *B. mori*, *P. xylostella*, and *T. ni* (Table 2.6) Comparisons between the acetylcholinesterases (ACE) (Figure 2.4, pink clade), neuroligins, and gliotactins (GLI) (Figure 2.4, grey clade) revealed 1:1:1:1 orthologs for all species (Tables 2.7-2.9). AtraACE2 shared 22.5% identity with AtraACE1 and 92.8 – 94.5 with Bmace1, Px003736, and TnCOE22. AtraACE1 was 56.2 – 59.4% similar to its respective orthologs. The gliotactin from *A. transitella* was 59.0 – 77.8% identical to other orthologs in the subfamily from each species. The separation of the neurotactins in *A. transitella*, *B. mori*, and *T. ni* into two clades with 1:1:1 orthologs was reflected by the alignments of the entire subfamily. AtraNRT1 was 67.5 – 76.0% identical to Bmnrt2 and TnCOE12 but shared

only 17.8 – 18.4% identity with AtraNRT2, Bmnrt2, and TnCOE82. AtraNRT2 shared 40 – 55.1% identity with Bmnrt1 and TnCOE82 but only 18.0 – 19.0% with Bmnrt2, AtraNRT1, and TnCOE12. *P. xylostella* did not have any neurotactins present in these two clades.

Phylogeny of *A. transitella* COEs

The placement of *A. transitella* COEs into their respective subfamilies was further analyzed through the individual species phylogeny (Figure 2.6). All subfamily assignments based on the lepidopteran phylogeny formed clades with their respective members. I recovered a clade with high support containing the neurodevelopmental class subfamilies, which included neuroligins (NLG), acetylcholinesterases (ACE), a gliotactin (GLI), an uncharacterized esterase (UN), and a neurotactin (NRT). The secreted catalytic class members juvenile hormone esterases (JHE), beta-esterases (BE), and integument esterases (IE) formed separate clades but with low support for their placements in the phylogeny. Supports within the alpha-esterases (AE) were highly variable, and I could not determine deeper relationships that connect this subfamily to others present in the phylogeny. The presence of substitutions in the catalytic triad were tracked as indicators of noncatalytic functions for each COE. I recovered a noncatalytic clade of alpha-esterases containing AtraAE9, AtraAE10, AtraAE11, and AtraAE12 with high support, similar to the alpha-esterases of the *B. mori* phylogeny (Yu *et al.* 2009). In total, there were 23 COEs with at least one substitution in the catalytic triad, 13 of which were placed in the alpha-esterase subfamily. Much like the lepidopteran phylogeny of COEs, I recovered AtraNRT2 in a separate clade from AtraNRT1.

DISCUSSION

With the exception of the 74 COEs described in the Colorado potato beetle (Lu *et al.* 2015), the four lepidopteran species used in our phylogeny have the largest number of COEs among insects with annotated genomes. These results showcase an expansion of the alpha-esterases (≥ 67% of all COEs) in lepidopteran genomes relative to insects in other orders. For example, *B. mori* and *T. ni* have more alpha-esterases in their genomes than the total COEs of dipterans and hymenopterans, including *Aedes aegypti*, *Anopheles gambiae*, *Anopheles sinensis*, *Apis mellifera*, *Culex pipiens quinquefasciatus*, *Drosophila. melanogaster*, *Musca domestica*, *Nasonia vitripennis*, and *Tribolium castaneum*. This phylogeny, presenting the fully-annotated suites of carboxylesterases from four lepidopteran species, is among the largest to date for any group of insects; however, the lack of bootstrap support for relationships among the alpha-esterases prevented me from adopting the Oakeshott *et al.* (2005, 2010) classification. Although the *A. transitella* COE phylogeny provided additional support for subfamily placements of juvenile hormone esterases, integument esterases, acetylcholinesterases, and neuroligins, the same problem occurred with respect to alpha-esterase relationships.

Orthologs were identifiable in these four lepidopteran species across most of the conserved subfamilies in the secreted catalytic and neurodevelopmental classes, including the beta-esterases, acetylcholinesterases, neuroligins, gliotactins, and neurotactins. The presence of orthologs in these genes with more conserved functions suggests they may have been generated through duplication events and subjected to subsequent purifying selection following speciation (Wu et al 2018). However, there was high variation in the number of JHEs present in these four species, with seven potential members identified in *P. xylostella* compared to four in *B. mori* and *T. ni*, and two in *A. transitella*. Gu *et al.* (2015) identified 11 potential JHEs in the *P. xylostella*

genome, seven of which are present as a *P. xylostella* JHE clade in our phylogeny. Among the 11 candidate JHEs, only Px004817 was recognized as a true JHE gene in *P. xylostella* (Gu *et al.* 2015, Duan *et al.* 2016). Similarly, of the putative JHEs characterized in the *B. mori* genome by Yu *et al.* (2009) and Tsubota *et al.* (2010), only one functioned as a juvenile hormone-specific degradation enzyme. Although JHEs appear to be reduced in *A. transitella* relative to the other lepidopterans, JH-specific metabolism may be similar if esterases classified as JHEs in *B. mori*, *P. xylostella*, and *T. ni* have evolved other functions.

Unexpectedly, neurotactins split into two different clades in the lepidopteran phylogeny. One of the two neurotactin clades recovered contained three genes (AtraNRT2, Bmnrt1, and TnCOE82) with the Ser-Acid-His triad intact. This was unexpected because esterases in the neurodevelopmental class in general lack enzymatic function and have at least one substitution in the triad (Darboux *et al.* 1996, Gilbert and Auld 2005). Neurotactins participate in axon guidance during embryonic and post-embryonic development (Gilbert and Auld 2005). Neurotactins may perform additional functions, or placement as neurotactins in this phylogeny warrants further evaluation because the bootstrap support for the AtraNRT2, Bmnrt1, and TnCOE82 clade is questionable as part of the neurodevelopmental class of esterases and as a sister group to the uncharacterized clade AtraUN2, Bmun2, TnCOE2, and TnCOE3. Previous phylogenies for *B. mori* and *A. sinensis* have split neurotactins across multiple clades at the base of their trees, but cluster them all together as neurotactins even though their placements are not supported. The placement of neurotactins into separate clades in the lepidopteran phylogeny, the *B. mori* phylogeny, and *Anopheles sinensis* phylogeny suggest that the neurotactin subfamily may require further functional investigation in order to place all neurotactins as true representatives of the same subfamily of esterases.

Based on the relative conservation of the majority of COEs in the secreted and neurodevelopmental classes, the full complements of COEs in lepidopterans appear to be dependent on the size of the alpha-esterases linked to dietary and detoxification functions. This phylogeny revealed one unique clade for *B. mori* and two for *T. ni* among the alpha-esterases, indications of unique duplications occurring after speciation (Ranson *et al.* 2002, Calla *et al.* 2017). *B. mori* and *T. ni* are more closely related in the lepidopteran phylogeny of Mitter *et al.* (2017) than any of the other species examined in this study, and these unique radiations in each species may have resulted from duplications of a shared gene in Macroheterocerae. Phylogenetic comparisons among *Am. transitella*, *B. mori*, *P. xylostella*, and *T. ni* COEs present several 1:1:1:1 orthologs but no unique expansions in the alpha-esterases for *A. transitella*.

The fact that the monophagous *B. mori*, restricted to foliage of white mulberry *(Morus alba)*, has a comparatively large COE inventory runs counter to the expectation that expansions characterize polyphagous species. *A. transitella* and *T. ni* are both highly polyphagous species but *T. ni* has 17 more COEs, excluding fragments, in its genome than does the genome of *Am. transitella*, 12 of which are classified as alpha-esterases. Calla *et al.* (2017) compared the total cytochrome P450 monooxygenase (P450s) complements (CYPome) and subfamily distributions across seven species of Lepidoptera, including *Am. transitella*, *B. mori*, and *P. xylostella*, and found an association between diversity of subfamilies and dietary complexity. Similar conclusions cannot be made based on the COE enzyme superfamily in these species of Lepidoptera. Carboxylesterases are placed into generalized classes and subfamilies based on phylogenetic relationships and arbitrarily named based on the clades in which they are located, whereas P450s have a strict nomenclature based on amino acid identities, with specific known functions at the subfamily level (in some cases) in insects across all orders. For now, conclusions

involving alpha-esterase relationships among species are limited; greater depth in the classification of COEs and increased functionality studies of the enzyme superfamily may be required before evolutionary patterns can be discerned.

Among the lepidopteran alpha-esterases, I recovered two clades comprising COEs that may have lost their catalytic functions because they contain at least one substitution in the catalytic triad. Each species has six COEs in these clades, except for *P. xylostella* which has seven, and 1:1:1:1 orthologs are identifiable in each clade. Yu *et al.* (2009) found 15 of 55 alpha-esterases in *B. mori* contained at least one substitution in the catalytic triad, and the authors suggested a role in sequestration of secondary metabolites in mulberry leaves for these COEs because they were detected in EST/microarray analysis in tissues such as fat body, midgut, and Malpighian tubules. Six of these noncatalytic COEs share orthologs with *A. transitella*, *P. xylostella,* and *T. ni* in these two unique clades, suggesting that these genes may be performing similar roles in each species. The number of substitutions in the catalytic triad of alpha-esterases was higher in all lepidopterans examined in our phylogeny relative to other insects described (e.g., *Anopheles sinensis*, *Anopheles gambiae*, and *Drosophila melanogaster*) (Wu *et al.* 2018), indicating potentially higher losses of function in Lepidoptera and/or acquisitions of noncatalytic roles.

COEs may confer resistance to multiple classes of insecticides, including organophosphates, carbamates, and pyrethroids, through mechanisms such as mutations in the active site, gene amplification, constitutive overexpression, and induction by substrates (Alon *et al.* 2008, Ciu *et al.* 2011, Wu *et al.* 2011). The complete annotation of the COEs in *Am. transitella* provides an invaluable resource for identifying genes involved in resistance to pyrethroids and the detoxification of the multitude of insecticide classes applied to manage *Am.*

transitella if cross-resistance develops between pyrethroids and other registered classes for *Am. transitella* control. Results from COE surveys conducted through RNA-seq and/or qPCR approaches to measure expression differences in pyrethroid-resistant populations of *A. sinensis* and *M. domestica* have revealed alpha-esterases, beta-esterases, integument esterases, acetylcholinesterases, a neurotactin, and glutactins as candidate resistance genes (Feng *et al.* 2018, Wu *et al.* 2018). Although the alpha-esterases may be the most likely potential candidates, if any, contributing toward pyrethroid resistance in *A. transitella*, it remains necessary to examine all subfamilies. This research provides the necessary foundation for further exploration into the role of COEs in the detoxification of xenobiotics in *A. transitella*.

TABLES AND FIGURES

Table 2.1. Carboxylesterase name, sequence length, identifiers, locations, and catalytic triad residues for *A. transitella*. AE: alpha-esterase; JHE: juvenile hormone esterase; BE: beta-esterase; IE: integument esterase; ACE: acetylcholinesterase; NLG: neuroligin; NRT: neurotactin; GLI: gliotactin; UN: uncharacterized esterase

COE Name	Length (amino acids)	NCBI Reference Sequence	Scaffold	Catalytic Triad
AtraAE1	562	XP_013183084.1	NW_013535351.1	GCSAG - E - H
AtraAE2	536	XP_013183096.1	NW_013535351.1	GCSAG - E - H
AtraAE3	634	XP_013183727.1	NW_013535317.1	GESAG - E - H
AtraAE4	559	XP_013183908.1	NW_013535363.1	GYSAG - E - H
AtraAE5	541	XP_013185290.1	NW_013535380.1	GESAG - E - H
AtraAE6	561	XP_013185325.1	NW_013535380.1	GISAG - E - H
AtraAE7	709	XP_013185425.1	NW_013535384.1	GESCG - E - H
AtraAE8	523	XP_013185431.1	NW_013535384.1	GESAG - E - H
AtraAE9	456	XP_013187418.1	NW_013535408.1	GSEKS - E - A
AtraAE10	582	XP_013187492.1	NW_013535408.1	GSDGG - E - I
AtraAE11	592	XP_013187500.1	NW_013535408.1	GNRGG - E - I
AtraAE12	586	XP_013187503.1	NW_013535408.1	GVQGG - E - S
AtraAE13	550	XP_013187563.1	NW_013535408.1	GHGSG - E - H
AtraAE14	586	XP_013187844.1	NW_013535412.1	GESAG - E - H
AtraAE15	663	XP_013188770.1	NW_013535437.1	GHGSG - E - H
AtraAE16	538	XP_013188943.1	NW_013535442.1	GQGSG - E - N
AtraAE17	555	XP_013189497.1	NW_013535319.1	GESAG - E - H
AtraAE18	561	XP_013190276.1	NW_013535456.1	GSNSG - E - Y
AtraAE19	531	XP_013190631.1	NW_013535463.1	GISAG - E - H
AtraAE20	468	XP_013190632.1	NW_013535463.1	GISAG - E - H
AtraAE21	533	XP_013191613.1	NW_013535491.1	GESYG - E - H
AtraAE22	546	XP_013192221.1	NW_013535505.1	GYSVG - E - H

Table 2.1 (continued)

AtraAE23	546	XP_013192657.1	NW_013535526.1	GESAG - E - H
AtraAE24	543	XP_013192658.1	NW_013535526.1	GESAG - E - H
AtraAE25	539	XP_013192666.1	NW_013535526.1	GESAG - E - H
AtraAE26	567	XP_013193078.1	NW_013535549.1	GYSAG - E - H
AtraAE27	563	XP_013193080.1	NW_013535549.1	GASAG - E - H
AtraAE28	532	XP_013193629.1	NW_013535577.1	GESAG - E - H
AtraAE29	546	XP_013193645.1	NW_013535577.1	GESAG - E - H
AtraAE30	540	XP_013193648.1	NW_013535577.1	GESAG - E - H
AtraAE31	560	XP_013196054.1	NW_013535793.1	GCSAG - E - H
AtraAE32	530	XP_013196080.1	NW_013535795.1	GQDAG - E -H
AtraAE33	464	XP_013196718.1	NW_013535925.1	GESAG - E - H
AtraAE34	558	XP_013196719.1	NW_013535925.1	GESAG - E - H
AtraAE35	564	XP_013196994.1	NW_013536093.1	GSSSG - E - H
AtraAE36	452	XP_013197465.1	NW_013536293.1	GVGAG - - - D
AtraAE37	462	XP_013197466.1	NW_013536293.1	GQGFG - E - F
AtraAE38	735	XP_013197469.1	NW_013536293.1	GDGTS - A - R
AtraAE39	544	XP_013200356.1	NW_013535312.1	GESAG - E - H
AtraAE40	551	XP_013201054.1	NW_013535347.1	EYSVA - E - V
AtraAE41	546	XP_013201071.1	NW_013535347.1	GESAG - E - H
AtraAE42	559	XP_013201150.1	NW_013535347.1	GASAG - D - H
AtraAE43	545	XP_013193084.1	NW_013535549.1	GYSAG - E - -
AtraAE44	646	XP_013192629.1	NW_013535526.1	GQSAG - E - H
AtraAE45	541	XP_013192630.1	NW_013535526.1	GQSAG - E - H
AtraJHE1	406	XP_013190943.1	NW_013535468.1	GQSAG - E - H
AtraJHE2	590	XP_013197467.1	NW_013536293.1	GQSAG - E - H
AtraIE1	510	XP_013187979.1	NW_013535420.1	GCSAG - E - H

Table 2.1 (continued)

AtraIE2	527	XP_013194783.1	NW_013535658.1	GCSAG - E - H
AtraBE1	564	XP_013189471.1	NW_013535443.1	GESAG - E - H
AtraBE2	559	XP_013196418.1	NW_013535848.1	GCSAG - E - H
AtraACE1	1026	XP_013196191.1	NW_013535808.1	GESAG - E - H
AtraACE2	646	XP_013190251.1	NW_013535456.1	GESAG - E - H
AtraGLI	872	XP_013183898.1	NW_013535363.1	GAGAG - E - F
AtraNLG1	761	XP_013189542.1	NW_013535445.1	GHAAG - E - T
AtraNLG2	569	XP_013192628.1	NW_013535525.1	GHGTG - E - H
AtraNLG3	994	XP_013192935.1	NW_013535542.1	GHGSG - E - H
AtraNLG4	1465	XP_013193013.1	NW_013535547.1	GHGRG - E - N
AtraNLG5	951	XP_013197753.1	NW_013536538.1	GHGSG - - - H
AtraNLG6	982	XP_013200418.1	NW_013535339.1	GHGTG - E - H
AtraNRT1	749	XP_013185565.1	NW_013535386.1	GHRAG - S - I
AtraNRT2	528	XP_013191167.1	NW_013535482.1	GHSAG - E - H
AtraUN1	761	XP_013196439.1	NW_013535851.1	GQGSG - E - H
AtraUN2	814	XP_013200959.1	NW_013535344.1	GHEAG - E - H

Table 2.2. Carboxylesterase distributions by class and subfamily based on phylogeny results for *A. transitella* (navel orangeworm), *T. ni* (cabbage looper), *B. mori* (silkmoth), and *P. xylostella* (diamondback moth).

Class	**Subfamily**	***A. transitella***	***T. ni***	***B. mori***	***P. xylostella***
Intracellular catalytic class	Alpha-esterase	45	52	55	40
Secreted catalytic class	Juvenile Hormone Esterase	2	4	4	7
	Integument Esterase	2	3	2	1
	Beta-esterase	2	2	2	2
	Glutactin	0	0	0	0
Neurodevelopmental class	Acetylcholinesterase	2	2	2	2
	Uncharacterized	2	3	2	0
	Gliotactin	1	1	1	2
	Neuroligin	6	6	6	6
	Neurotactin	2	2	2	0
	Total	**64**	**75**	**76**	**60**

Table 2.3. Amino acid alignments for the juvenile hormone esterases (JHE) in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	Px011247	Px004818	Px012592	Px007180	Px001687	Px004817	Px009124	Bmjhe4	Bmjhe2	Bmjhe3	AtraJHE1	AtraJHE2	TnCOE48	Bmjhe1	TnCOE50	TnCOE51	TnCOE52
Px011247		35	35.9	38.1	38.1	44.3	92.8	35.3	35.1	34.2	25.5	38.9	37.1	40.4	36.2	36	36.2
Px004818	35		51	48.8	49	41.8	37.9	32.9	33.1	32.6	25.9	37.3	34.1	36	33.7	35.6	34.2
Px012592	35.9	51		60.9	60.5	42.8	38	36.5	35.9	34.8	28.1	39.3	38.8	38.7	33.8	38	36.5
Px007180	38.1	48.8	60.9		89.2	44.3	39.8	36	35.4	35.4	27.9	38.1	36.3	40.7	34.5	37.8	38
Px001687	38.1	49	60.5	89.2		44.4	40.2	35.3	34.5	34.2	27.7	37.8	36.2	41	34.1	36.4	37.3
Px004817	44.3	41.8	42.8	44.3	44.4		45.4	37	35.7	34.5	28.8	42.7	39.1	43.8	36.5	38.3	36.7
Px009124	92.8	37.9	38	39.8	40.2	45.4		37.3	36.9	35.6	27.3	41.2	40	43.2	38.1	37.5	37.8
Bmjhe4	35.3	32.9	36.5	36	35.3	37	37.3		72.1	71.6	32.1	42	40.1	42.5	43.4	44.4	43.2
Bmjhe2	35.1	33.1	35.9	35.4	34.5	35.7	36.9	72.1		74	30.7	40.8	38.2	41.5	43.4	44.5	44.6
Bmjhe3	34.2	32.6	34.8	35.4	34.2	34.5	35.6	71.6	74		30	39.2	38	41	41.9	44.5	45.2
AtraJHE1	25.5	25.9	28.1	27.9	27.7	28.8	27.3	32.1	30.7	30		70.3	34	35.4	31.4	31.3	32.6
AtraJHE2	38.9	37.3	39.3	38.1	37.8	42.7	41.2	42	40.8	39.2	70.3		48.9	50.3	45.1	43.9	44.9
TnCOE48	37.1	34.1	38.8	36.3	36.2	39.1	40	40.1	38.2	38	34	48.9		51.3	42.4	43.7	43.5
Bmjhe1	40.4	36	38.7	40.7	41	43.8	43.2	42.5	41.5	41	35.4	50.3	51.3		45.3	45	46.8
TnCOE50	36.2	33.7	33.8	34.5	34.1	36.5	38.1	43.4	43.4	41.9	31.4	45.1	42.4	45.3		48.1	48.9
TnCOE51	36	35.6	38	37.8	36.4	38.3	37.5	44.4	44.5	44.5	31.3	43.9	43.7	45	48.1		69.2
TnCOE52	36.2	34.2	36.5	38	37.3	36.7	37.8	43.2	44.6	45.2	32.6	44.9	43.5	46.8	48.9	69.2	

Table 2.4. Amino acid alignments for the beta-esterases (BE) in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	Px017920	Px011941	AtraBE2	Bmbe1	TnCOE26	AtraBE1	Bmbe2	TnCOE80
Px017920		32.7	33.4	33.6	34.3	62.5	65.5	67.3
Px011941	32.7		68.3	68.5	67.3	32.2	32.8	32
AtraBE2	33.4	68.3		70.5	67.1	32.2	31.4	33
Bmbe1	33.6	68.5	70.5		72.9	32.4	31.7	33.9
TnCOE26	34.3	67.3	67.1	72.9		31.5	31.7	32.2
AtraBE1	62.5	32.2	32.2	32.4	31.5		67.3	68.1
Bmbe2	65.5	32.8	31.4	31.7	31.7	67.3		69.9
TnCOE80	67.3	32	33	33.9	32.2	68.1	69.9	

Table 2.5. Amino acid alignments for the integument esterases (IE) in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	Px004284	TnCOE17	AtraIE1	Bmie2	Bmie1	TnCOE19	TnCOE18	AtraIE2
Px004284		23.2	42.6	28.4	28.1	28.7	28.6	45.4
TnCOE17	23.2		51.4	51.1	46.9	48.8	44.7	48.3
AtraIE1	42.6	51.4		63	58.9	62.7	59.1	63.4
Bmie2	28.4	51.1	63		57.1	61.4	57.3	64.7
Bmie1	28.1	46.9	58.9	57.1		60.1	64.1	65.1
TnCOE19	28.7	48.8	62.7	61.4	60.1		62.5	67.6
TnCOE18	28.6	44.7	59.1	57.3	64.1	62.5		65.8
AtraIE2	45.4	48.3	63.4	64.7	65.1	67.6	65.8	

Table 2.6. Amino acid alignments for the neuroligins (NLG) *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	Px015105	AtraNLG4	Px000187	Bmnlg3	TnCOE74	Px001207	Bmnlg1	AtraNLG1	TnCOE23	Px001733	Bmnlg2	AtraNLG6	TnCOE24	Bmnlg6	Px007852	AtraNLG2	TnCOE75	Px013955	Bmnlg4	AtraNLG3	TnCOE72	AtraNLG5	Bmnlg5	TnCOE73
Px015105		24.3	23.5	21.3	22.4	23.6	25	25.6	25.1	22.8	26.1	26.7	26.4	29.8	31.9	32.6	32.2	36.6	29.2	35.8	37.2	56.5	51.1	52.2
AtraNLG4	24.3		65.7	64.2	68.7	19.9	25.2	21.3	25	19.7	23.1	22.7	23.4	24.9	24.1	36.9	25	23.7	20.8	24.2	24.6	21.6	23.2	23.4
Px000187	23.5	65.7		65.7	67.8	20.1	24.3	20.9	24.4	18.7	22.9	22.4	23.4	25.2	24	37.9	25.5	23.4	20.7	23.6	24.2	20.8	22.2	22.7
Bmnlg3	21.3	64.2	65.7		70.1	22.2	23.1	23.9	23.7	18.1	25.1	25.4	25.6	26.6	26.6	34.9	27.5	24.9	21.4	24.7	24.8	21.9	23.5	24.3
TnCOE74	22.4	68.7	67.8	70.1		19.3	24.4	20.2	23.9	19.8	22.9	22.7	23.4	24.5	24.4	37.6	25.5	23	20.7	23.7	24	20.7	22.1	22.7
Px001207	23.6	19.9	20.1	22.2	19.3		63.5	60.7	64	24.4	30.1	29.7	30.6	27.6	28.3	38	28.4	26.9	21.4	26.8	26.6	24	25.6	25.9
Bmnlg1	25	25.2	24.3	23.1	24.4	63.5		72.7	73	28.5	34.8	35.1	35.7	33.9	32.9	38	33.9	32.7	27.5	32.8	33.1	29.2	31	30.9
AtraNLG1	25.6	21.3	20.9	23.9	20.2	60.7	72.7		82.8	26	33.2	32.4	34.5	30.8	32.1	39.9	32.9	32	27	31.4	31.5	27.1	29	28.8
TnCOE23	25.1	25	24.4	23.7	23.9	64	73	82.8		25.2	35.8	35.7	36.7	33.1	33.6	39.8	34.1	34.1	28.2	33.3	33.8	29.9	31.9	31.9
Px001733	22.8	19.7	18.7	18.1	19.8	24.4	28.5	26	25.2		51.4	52.1	52.6	25	23.3	36	24	25.2	25	24.7	24.8	22.3	24.4	25.4
Bmnlg2	26.1	23.1	22.9	25.1	22.9	30.1	34.8	33.2	35.8	51.4		86.8	88.2	29.1	29.4	40.2	30.3	32.6	27.5	31.7	32.1	29.6	31.7	31.7
AtraNLG6	26.7	22.7	22.4	25.4	22.7	29.7	35.1	32.4	35.7	52.1	86.8		88.1	29.2	29.6	40	30.1	32.5	28.2	32.3	32.8	29.5	31.8	31.1
TnCOE24	26.4	23.4	23.4	25.6	23.4	30.6	35.7	34.5	36.7	52.6	88.2	88.1		29.7	30.4	40.8	30.8	34.3	29.3	33.4	34	29.4	32	31.4
Bmnlg6	29.8	24.9	25.2	26.6	24.5	27.6	33.9	30.8	33.1	25	29.1	29.2	29.7		67.7	76.4	69.7	36.8	33.1	36.6	36.6	34.2	35.4	35.8
Px007852	31.9	24.1	24	26.6	24.4	28.3	32.9	32.1	33.6	23.3	29.4	29.6	30.4	67.7		85.3	84.2	40.5	35.2	39.8	39.5	35.5	39.2	39.2
AtraNLG2	32.6	36.9	37.9	34.9	37.6	38	38	39.9	39.8	36	40.2	40	40.8	76.4	85.3		94.2	49.7	41.8	49.7	50.1	42.9	48.7	49
TnCOE75	32.2	25	25.5	27.5	25.5	28.4	33.9	32.9	34.1	24	30.3	30.1	30.8	69.7	84.2	94.2		40.5	35.8	40.5	40.4	35.6	39.1	39.5
Px013955	36.6	23.7	23.4	24.9	23	26.9	32.7	32	34.1	25.2	32.6	32.5	34.3	36.8	40.5	49.7	40.5		77	86.5	83.8	47.3	52.4	53.4
Bmnlg4	29.2	20.8	20.7	21.4	20.7	21.4	27.5	27	28.2	25	27.5	28.2	29.3	33.1	35.2	41.8	35.8	77		81.9	80.9	42.4	47.3	48.5
AtraNLG3	35.8	24.2	23.6	24.7	23.7	26.8	32.8	31.4	33.3	24.7	31.7	32.3	33.4	36.6	39.8	49.7	40.5	86.5	81.9		89.9	46.4	52.1	52.6
TnCOE72	37.2	24.6	24.2	24.8	24	26.6	33.1	31.5	33.8	24.8	32.1	32.8	34	36.6	39.5	50.1	40.4	83.8	80.9	89.9		46.5	52.2	52.8
AtraNLG5	56.5	21.6	20.8	21.9	20.7	24	29.2	27.1	29.9	22.3	29.6	29.5	29.4	34.2	35.5	42.9	35.6	47.3	42.4	46.4	46.5		74.9	74
Bmnlg5	51.1	23.2	22.2	23.5	22.1	25.6	31	29	31.9	24.4	31.7	31.8	32	35.4	39.2	48.7	39.1	52.4	47.3	52.1	52.2	74.9		84.5
TnCOE73	52.2	23.4	22.7	24.3	22.7	25.9	30.9	28.8	31.9	25.4	31.7	31.1	31.4	35.8	39.2	49	39.5	53.4	48.5	52.6	52.8	74	84.5	

Table 2.7. Amino acid alignments for the acetylcholinesterases (ACE) in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	Bmace2	Bmace1	Px003736	TnCOE22	AtraACE2	Px008913	AtraACE1	TnCOE71
Bmace2		33.2	33.2	33.2	32.5	81.4	56.2	86.9
Bmace1	33.2		91.7	93.4	93.3	33.1	22.5	32.5
Px003736	33.2	91.7		94.4	92.8	33.6	22.7	32.6
TnCOE22	33.2	93.4	94.4		94.5	33.5	23.1	32.9
AtraACE2	32.5	93.3	92.8	94.5		32.8	22.5	32
Px008913	81.4	33.1	33.6	33.5	32.8		55.7	84.2
AtraACE1	56.2	22.5	22.7	23.1	22.5	55.7		59.4
TnCOE71	86.9	32.5	32.6	32.9	32	84.2	59.4	

Table 2.8. Amino acid alignments for the gliotactins (GLI) in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	Bmgli	AtraGli	TnCOE27	Px006690	Px010105
Bmgli		73.4	59.7	79.6	79.0
AtraGli	73.4		59.0	77.8	75.8
TnCOE27	59.7	59.0		62.0	63.1
Px006690	79.6	77.8	62.0		97.3
Px010105	79.0	75.8	63.1	97.3	

Table 2.9. Amino acid alignments for the neurotactins (NRT) in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	AtraNRT2	Bmnrt2	AtraNRT1	TnCOE12	Bmnrt1	TnCOE82
AtraNRT2		19.0	18.0	18.4	40.0	55.1
Bmnrt2	19.0		67.5	70.9	19.2	19.0
AtraNRT1	18.0	67.5		76.0	18.4	17.8
TnCOE12	18.4	70.9	76.0		19.7	18.2
Bmnrt1	40.0	19.2	18.4	19.7		43.0
TnCOE82	55.1	19.0	17.8	18.2	43.0	

Table 2.10. Amino acid alignments for the uncharacterized esterases in *Amyelois transitella* (Atra), *Bombyx mori* (Bm), *Plutella xylostella* (Px), and *Trichoplusia ni* (Tn).

	TnCOE3	AtraUN2	Bmun2	TnCOE2	AtraUN1	Bmun1	TnCOE4
TnCOE3		39.8	43.0	61.4	15.1	13.9	14.3
AtraUN2	39.8		58.0	41.0	17.4	17.8	17.3
Bmun2	43.0	58.0		57.4	18.8	18.6	19.2
TnCOE2	61.4	41.0	57.4		16.1	16.7	16.1
AtraUN1	15.1	17.4	18.8	16.1		52.8	49.4
Bmun1	13.9	17.8	18.6	16.7	52.8		64.3
TnCOE4	14.3	17.3	19.2	16.1	49.4	64.3	

Figure 2.1. Gene models for the alpha-esterases in *A. transitella*. Black bars represent exons and lines as introns. The numbers indicate the splicing phases of the COE genes (0: phase 0; 1: phase 1; 2: phase 2).

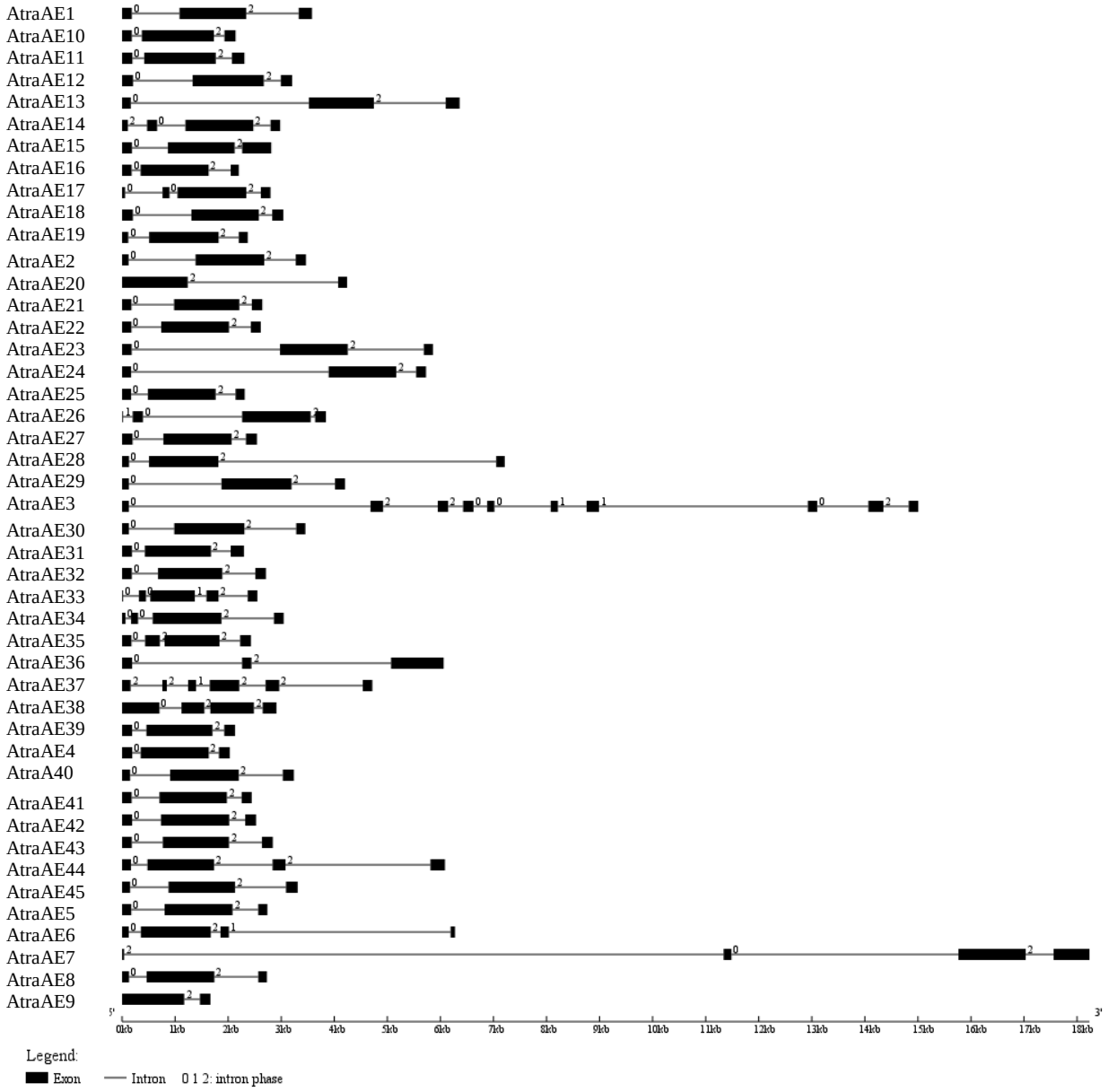


Figure 2.2. Gene models for the secreted catalytic class of COEs which include the beta-esterases (BE), integument esterases (IE), juvenile hormone esterases (JHE). Black bars represent exons and lines as introns. The numbers indicate the splicing phases of the COE genes (0: phase 0; 1: phase 1; 2: phase 2).

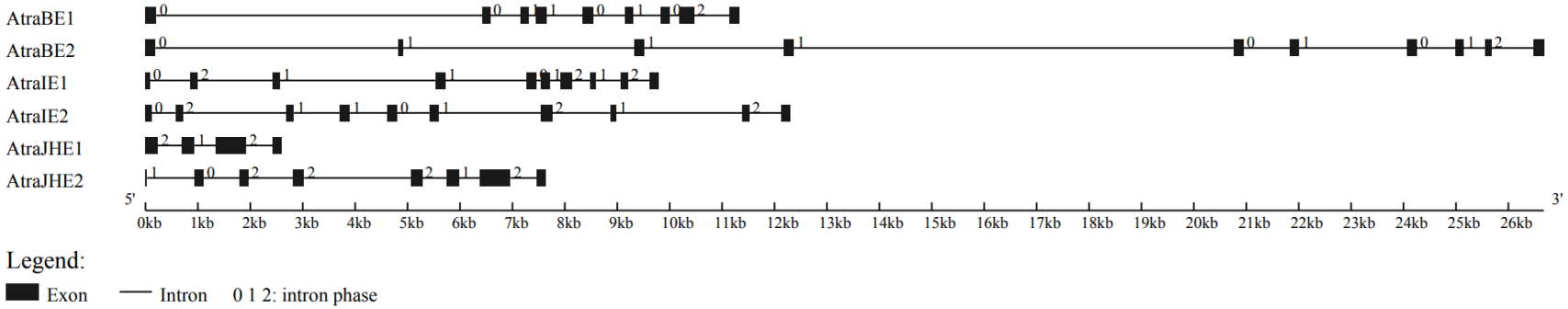


Figure 2.3. Gene models for the neurodevelopmental class of COEs which include the acetylcholinesterases (ACE), gliotactin (GLI), neuroligins (NLG), neurotactins (NRT), and uncharacterized esterases (UN). Black bars represent exons and lines as introns. The numbers indicate the splicing phases of the COE genes (0: phase 0; 1: phase 1; 2: phase 2).

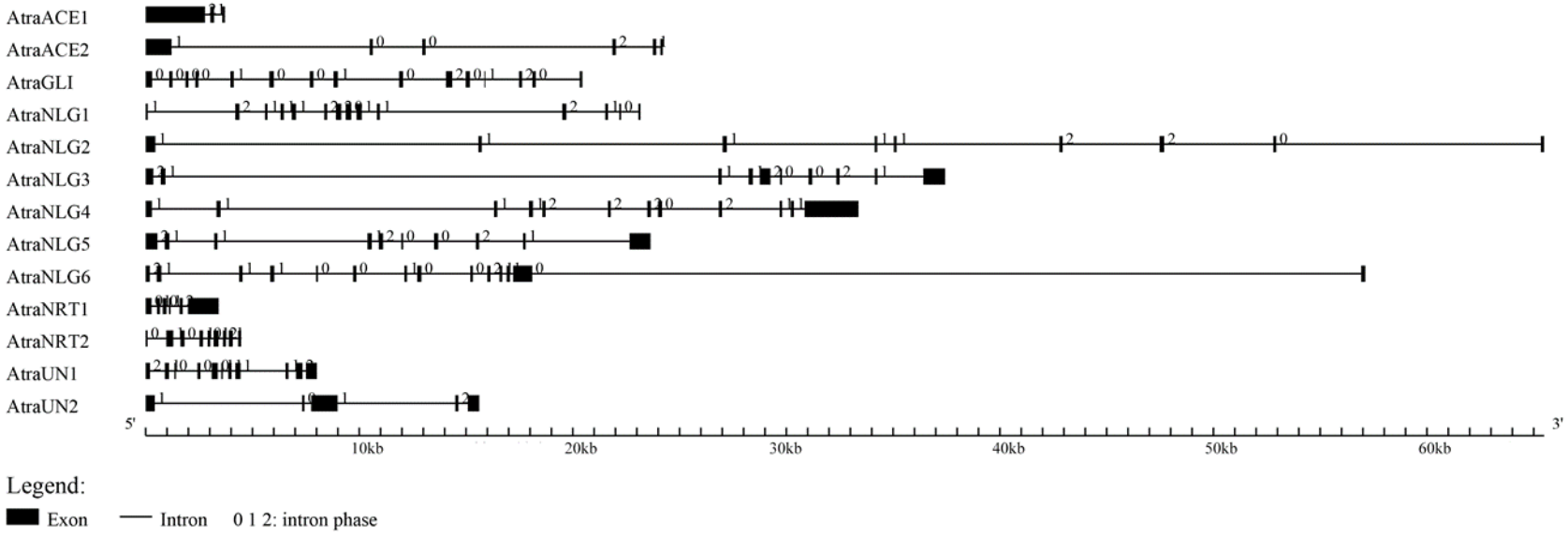


Figure 2.4. Lepidopteran phylogeny for *A. transitella* (orange text), *B. mori* (blue), *P. xylostella* (purple), and *T. ni* (green). Bootstrap values ≥ 50 are presented. Highlighted clades indicate functional subfamilies of COEs (left to right – green: juvenile hormone esterases; blue: beta-esterases; yellow: integument esterases; red: neuroligins; purple: uncharacterized esterases; orange: neurotactins; grey: gliotactins; pink: acetylcholinesterases; orange: neurotactins). All remaining clades contain the alpha-esterases.

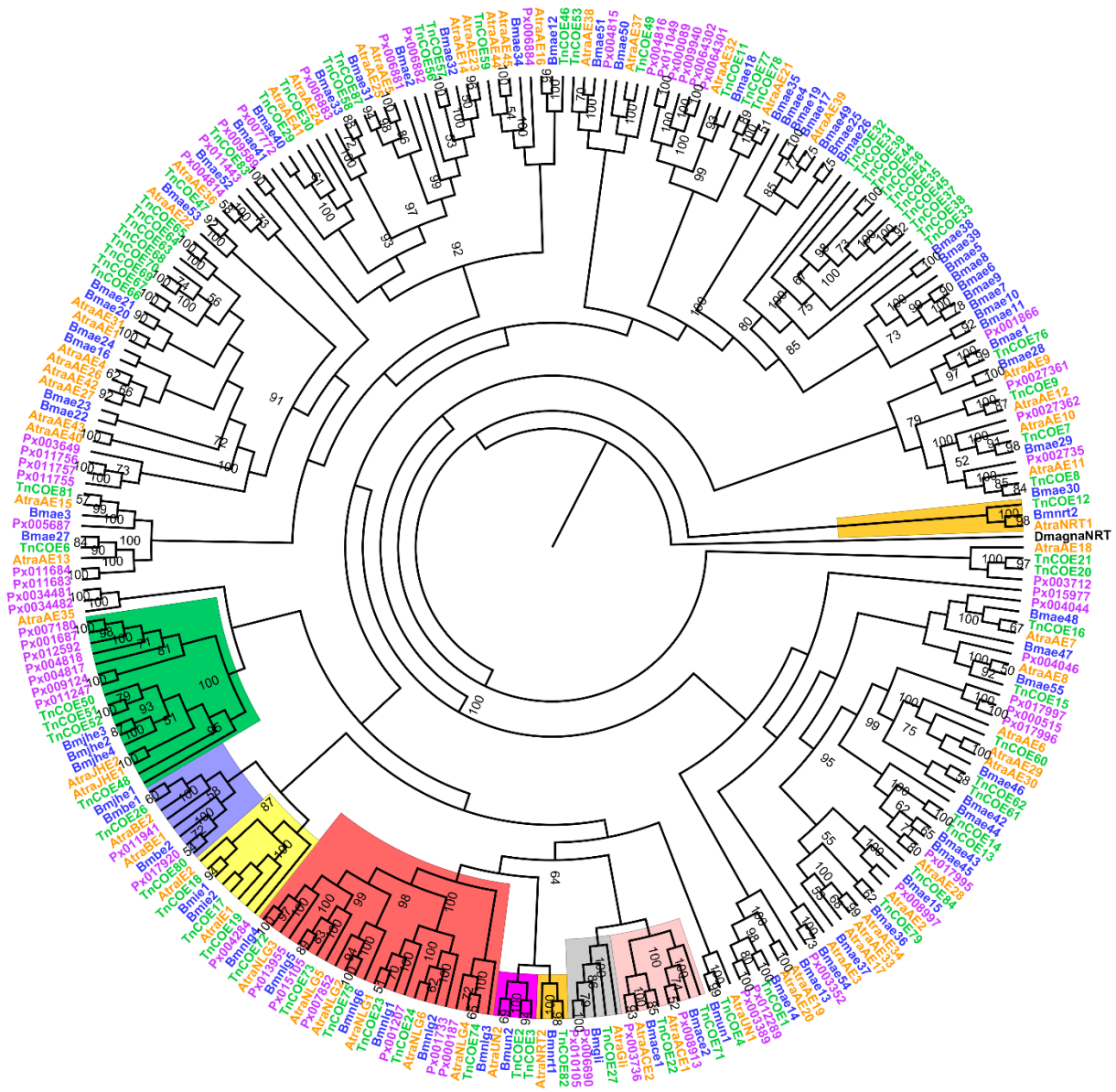


Figure 2.5. Lepidopteran phylogeny for *A. transitella* (orange text), *B. mori* (blue), *P. xylostella* (purple), and *T. ni* (green). Bootstrap values ≥ 50 are presented. Purple clades highlighted consist of alpha-esterases from each species that have at least one substitution in the triad, indicating a potential loss of catalytic function.

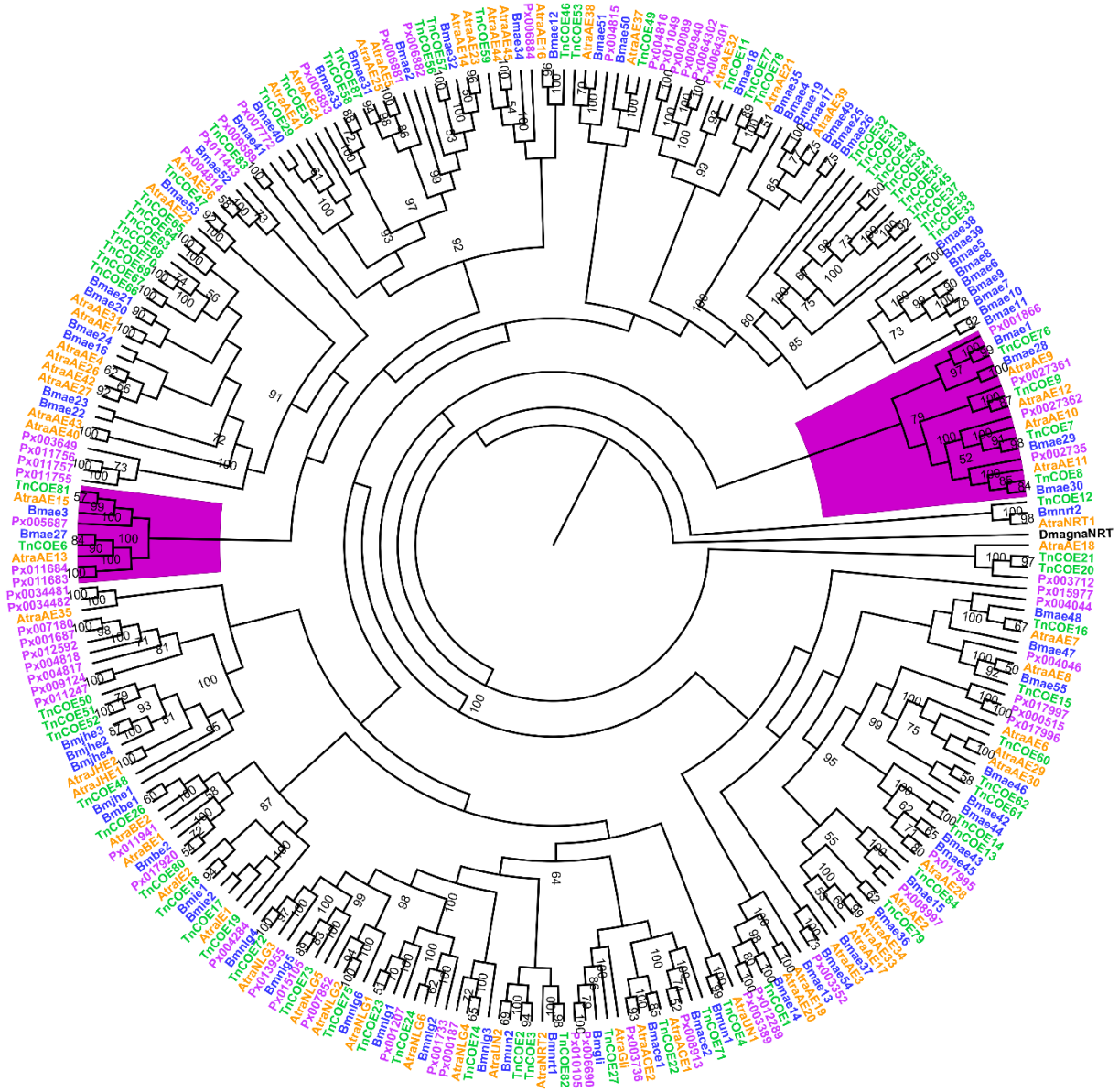
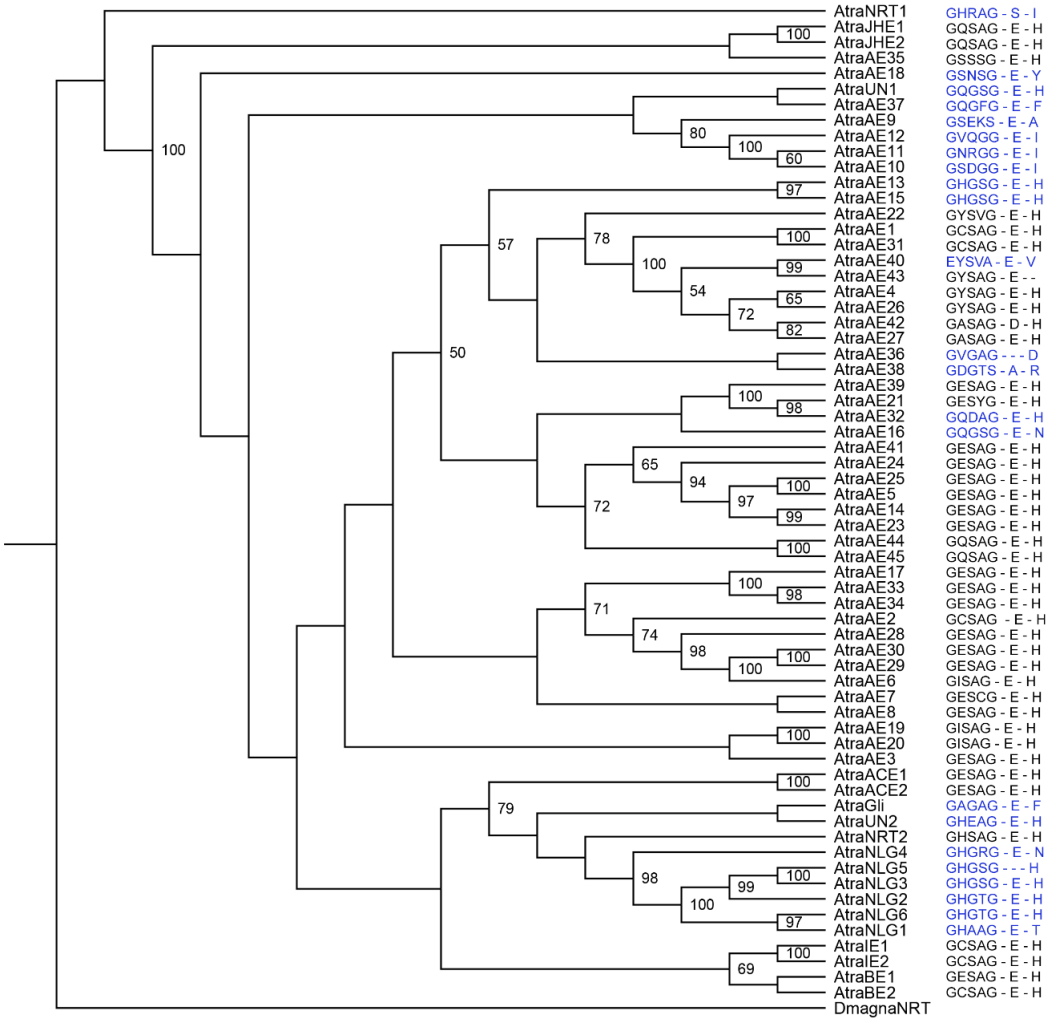


Figure 2.6. Phylogeny of 64 carboxylesterases in *Amyelois transitella* with *Daphnia magna* as the outgroup. Bootstrap values ≥ 50 are presented. AE: alpha-esterase; JHE: juvenile hormone esterase; BE: beta-esterase; IE: integument esterase; ACE: acetylcholinesterase; NLG: neuroligin; NRT: neurotactin; GLI: gliotactin; UN: uncharacterized esterase. COEs with one or more substitutions in the catalytic triad GxSxG-E/D-H are displayed in blue text.



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CHAPTER 3

A POINT MUTATION ASSOCIATED WITH PYRETHROID RESISTANCE IN THE NAVEL ORANGEWORM (*AMYELOIS TRANSITLLA*) AND THE HISTORY OF INSECTICIDE USE FOR NAVEL ORANGEWORM CONTROL

INTRODUCTION

The navel orangeworm (*Amyelois transitella*) is the most important economic pest of almonds and pistachios in California orchards. *A. transitella* damages tree nuts directly by feeding on kernels after the hulls split open and indirectly by serving as a vector for the aflatoxin-producing fungal pathogen *Aspergillus flavus* (Palumbo *et al.* 2014). Although primary control of *A. transitella* occurs through sanitation, insecticide applications have risen substantially to protect the ~ 7-billion-dollar value of these combined commodities and reduce damage to acceptable levels (Higbee and Siegel 2009, Niu *et al.* 2012, Demkovich *et al.* 2015a, NASS 2017). Pyrethroid insecticides have been the most frequently applied insecticides in almond and pistachio orchards, as measured through a combination of the number of applications, pounds applied, and number of acres treated (Demkovich *et al.* 2015a, CDPR 1990-2017). Not surprisingly, heavy use of pyrethroids facilitated the evolution of resistance in *A. transitella*, with the reported first case occurring in Kern County almond orchards in 2013.

The sequencing of the *A. transitella* genome through the i5k project (http://i5k.github.io/) provided an opportunity to expand the search for resistance mechanisms beyond the cytochrome P450s (P450s) previously implicated (Chapter 1) and locate regions of the genome under selection pressure from pyrethroid use in the resistant and susceptible strains. Calla *et al.* (in preparation) further elucidated mechanisms of pyrethroid resistance using a population genomics

approach through the pooled sequencing of individuals (Pool-seq, Schlötterer *et al.* 2014) with the genomes of the R347 and ALM populations (Chapter 1), in addition to a population of *A. transitella* collected from fig orchards in Madera County, where pyrethroids are not registered for use. These authors detected a vast (~1.3-Mb) selective sweep containing genes encoding two voltage-gated channel proteins, three cytochrome P450 monooxygenases and several transcription factors. One of the primary mechanisms of pyrethroid resistance among insects involves target-site resistance, and Calla *et al.* (in preparation) identified a well-described point mutation in the *para* gene, which encodes the target voltage-gated sodium channel (Khambay and Jewess 2005, Feyereisen 2011). This mutation, *kdr* (knockdown resistance), alters the conformation of the sodium channel and confers resistance to DDT and pyrethroid insecticides (Haddi *et al.* 2012). The *kdr* mutation was absent in the reference genome SPIRL-1966 but was unexpectedly shared by the population considered susceptible (ALM, Chapter 1), the resistant population (R347, Chapter 1), and the population collected from fig orchards, an indication that pyrethroid resistance was not restricted to Kern County or almond orchards in general.

Despite the absence of the *kdr* point mutation in SPIRL collected from almond orchards in 1966, the presence of the selective sweep in this reference genome population indicates that the region of the genome may have been under selection prior to the expansion of pyrethroid insecticide use. Limited records of insecticide use during this time suggest applications of DDT occurred throughout the Central Valley of California when *A. transitella* had established itself as a secondary pest to codling moth in walnuts (Ortega 1948, Michelbacher and Ross 1955, Michelbacher and Davis 1961, Cory *et al.* 1971). The earliest records of *A. transitella* infestation having a major economic impact occurred from 1977 to 1981, when *A. transitella* damage to kernels averaged 5.3%, but this infestation occurred well after DDT was banned in 1972 (Curtis

and Barnes 1977, Curtis *et al.* 1984). Because DDT targets the same sodium channel as do the pyrethroids, selection pressure from this insecticide may have created the selective sweep four decades ago; however, the unavailability of *A. transitella* specimens exposed to DDT selection and absence of detailed pesticide application records prevent a definitive test of this hypothesis.

To investigate the history of the *para* mutation discovered in the selective sweep, I used two approaches. Insecticide application records in California tree nuts after 1990 are available through the California Department of Pesticide Regulations (CDPR). Pesticide Use Report (PUR) data from CDPR provide an abundance of information concerning applications in California by commodity, counties, and specific active ingredients. In the context of *A. transitella* management, I located the records of registered insecticide classes for control, which include pyrethroids (Insecticide Resistance Action Committee (IRAC) subgroup 3A), organophosphates (IRAC subgroup 1B), diamides (IRAC Group 28), diacylhydrazine (IRAC Group 18), neonicotinoids (IRAC subgroup 4A), and spinosyns (IRAC Group 5) and examined their history of application in almond and pistachio orchards during the period from 2000 to 2016. I also expanded the genetic analysis of the sweep region geographically and temporally. I re-sequenced the region of the *para* gene using frozen larvae from the reference strain SPIRL-1966 in order to confirm its absence. Furthermore, I sampled a population from the northern counties of California where insecticide applications have historically been less intense relative to the San Joaquin Valley (where Pool-seq populations were collected), performed bioassays to assess resistance to DDT and bifenthrin, and checked for the point mutation in *para*.

MATERIALS AND METHODS

Insects

In 2011, I received eggs from the *A. transitella* strain SPIRL-1966 from Dr. Joel Siegel (USDA-ARS, Parlier, CA). After hatching, larvae were reared on a wheat bran diet (Finney and Brinkmann 1967) until fifth instar, at which point they were frozen at -20ºC. In 2018, these frozen SPIRL-1966 larvae were retrieved from the freezer where they had been stored and used for DNA extraction and sequencing. To sample northern populations, fifth instar larvae were collected from mummies in almond orchards in Yolo and Colusa Counties by collaborator Cris Wilk (Scientific Methods) in 2018 and shipped to the University of Illinois at Urbana-Champaign (UIUC) from Chico, CA (designated the CHICO strain of *A. transitella*) (Figure 3.1). In 2016, larvae from the Almond (ALM) strain of *A. transitella* were collected from almond orchards in Madera County from mummified fruits and shipped to UIUC by Joel Siegel (USDA-ARS, Parlier, CA). Also collected and shipped to UIUC by Joel Siegel in 2018 were larvae from the USDA laboratory CPQ colony of *A. transitella* (Siegel *et al.* 2010). The CPQ colony replaced SPIRL-1966 as the laboratory strain in 2012 after a mutation in the SPIRL colonies at the USDA-ARS caused larvae to produce excessive webbing and undergo an additional sixth instar instead of five (J. Siegel, personal communication). Resistant (R347) larvae were collected from mummy nuts in Kern County almond orchards and shipped by Brad Higbee (Trécé). The ALM, CHICO, and R347 strains were maintained in an incubator at the UIUC at temperatures of 28 ± 4ºC and photoperiod as a substrate for of 16:8 (L:D) h cycle. Adults were collected and placed in Mason jars with paper towels to serve oviposition. Eggs were collected every 48 h for use with insect bioassays.

DNA extraction and Sanger sequencing the *para* locus laboratory and CHICO strains

Because the reference genome strain SPIRL-1966 is no longer kept in colony at the ARS facility in Parlier CA, I extracted DNA from ten frozen whole-body fifth instar SPIRL-1966 larvae, using an E.Z.N.A.® insect DNA kit (Omega Bio-tek, Norcross, GA) following the manufacturer's instructions. I used existing midgut cDNA from ten samples of the CPQ strain. Existing stocks of cDNA from CPQ midguts were previously synthesized using a Protoscript II kit (NEB, Ipswich, MA) from fifth-instar larvae that had fed on semi-synthetic artificial diet (Waldbauer *et al.* 1984). The field population consisted of the CHICO strain collected from almond mummy nuts in Yolo and Colusa Counties. DNA was extracted from 20 whole-body fifth instar larvae using the same kit used to extract DNA from SPIRL-1966. I conducted PCR on all three strains using primers designed to flank the region of the *kdr* mutation in the *para* gene (Forward 5'- ACCAAGGTGGAACTTCACAGAT -3' Reverse 5'- AGCAATTTCAAGAAGTCAGCAACA -3'). PCR amplicons were sequenced (Eurofins Genomics, Louisville, KY) and sequences were aligned to the reference *para* sequence in Geneious software version 11.0.2 (http://www.geneious.com, Kearse *et al.* 2012) to verify the presence or absence of the mutation.

Insecticide bioassays:

In order to assess whether the *kdr* mutation confers resistance to insecticides targeting the sodium channel in *A. transitella*, I conducted a series of bioassays using DDT and bifenthrin to determine the median-lethal concentrations (LC_{50}) and then compare the susceptibility of the populations examined in this study. The LC_{50} values for bifenthrin and DDT in the sequenced strains were determined through feeding assays with semi-synthetic artificial diets (Waldbauer *et*

al. 1984, Demkovich *et al.* 2015a) containing insecticides mixed in at a range of concentrations. Bifenthrin (Chem Service Inc., West Chester, PA), or DDT (Sigma-Aldrich Co., St. Louis, MO) were stirred into the diet at different concentrations for each strain and poured into separate 1-oz (28 ml) cups to set. Treatments and concentrations were: DDT – ALM: 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm; DDT – R347: 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm; DDT – CPQ: 10 ppm, 20 ppm, 35 ppm, 50 ppm, 75 ppm, 100 ppm; bifenthrin – CHICO: 8 ppm, 16 ppm, 24 ppm, 36 ppm, 48 ppm, 54 ppm, 75 ppm. The LC_{50} values for bifenthrin in the ALM and R347 strains were identified in Chapter 1 but are presented in this Chapter to compare with the bifenthrin LC_{50} of CHICO, as well as DDT toxicity among the different strains. I also used the bifenthrin LC_{50} values reported in Bagchi *et al.* (2016) for CPQ. Four neonates were transferred with a soft brush into each plastic cup containing bifenthrin or methanol as the solvent control. Twenty larvae from each strain were exposed to their respective bifenthrin or DDT concentrations and each assay was replicated three times per concentration. Neonate mortality on diets was assessed after 48 h and scored according to a movement response after being touched by a soft brush. Probit analysis (SPSS version 22, SPSS Inc., Chicago, IL) was used to determine the LC_{50} values. Differences between populations were considered significant if their respective 95% confidence intervals in the Probit analysis did not overlap.

Pesticide Application Data:

Records of insecticide use were accessed through the California Department of Pesticide Regulation (CDPR) - pesticide use annual reports from 1990-2016. Insecticide use in almond and pistachio orchards was analyzed in Kern County, Madera County, and statewide based on number of applications, pounds of active ingredient, and acres treated from 2006 to 2016.

Insecticide records were pulled and separated by class to include pyrethroids, methoxyfenozide, diamides, and spinosyns. These classes were selected because they are the active ingredients most frequently used in *A. transitella* control (Higbee and Siegel 2012, Niu *et al.* 2012, Demkovich *et al.* 2015b). The pyrethroids included in the analysis were bifenthrin (Brigade WSB®, Fanfare®, Bifenture®), cyfluthrin, beta-cyfluthrin (Baythroid®), zeta-cypermethrin (Mustang®), (S)-cypermethrin, esfenvalerate (Asana®), fenpropathrin (Danitol®), lambda-cyhalothrin (LambdaCy®, Warrior®), and permethrin (PermUp®). Methoxyfenozide (Intrepid®) is applied on its own or as a mix with the spinosyn spinetoram (as Intrepid-Edge®). Spinosyn active ingredients chosen for analysis included spinetoram (Delegate®) and spinosad (Entrust®). The diamide active ingredients examined included chlorantranliprole (Altacor®) and flubendiamide (Belt®). I excluded organophosphates because the implementation of the 1996 Food Quality Protection Act resulted in the cancellation/restriction of multiple insecticides in this class, including azinphos-methyl (Guthion®), which was frequently applied prior to 1996 for *A. transitella* control (Higbee and Siegel 2012). The restriction of organophosphates resulted in a shift toward the insecticide classes listed above, which are the focus of this overview of trends in overall use.

In addition to applications, pounds of active ingredient, and acres treated for these insecticide classes, I adapted a measure from Liu *et al.* (2012) and Zhan *et al.* (2014) known as Usage Intensity (*UI*). Usage intensity is calculated as = Σ (pesticide use amount) / field area. Liu *et al.* (2012) and Zhan *et al.* (2014) listed this measure in kg / ha, but, because my analysis focused on averages and not individual applications, I did not make direct comparisons with *UI*s in these studies and left the units in my calculations as PUR provides them, pounds / treated acre. I compared bifenthrin use by pounds of active ingredient relative to all pyrethroids registered in

almond and pistachio orchards in Kern County, Madera County, and statewide. I also analyzed bifenthrin use for all registered products by trade name in almond and pistachio orchards from 2006 to 2017.

Data selection:

Although the DPR database has internal error checking procedures (described in Wilhoit *et al.* 2011), previous studies involving the Pesticide Use Reporting records have described the need to filter additional applications of pesticides due to duplications (e.g., the same active ingredient on the same area of a site on the same day), missing data (e.g., when the unit for treated areas is unknown), or non-compliant measurements (e.g., when the unit was measured in units other than square feet or acres) (Epstein *et al*. 2001, Zhang *et al.* 2005, Liu *et al.* 2012). For example, in their environmental risk assessment of organophosphate and pyrethroid use in almond orchards from 1992-2005, Liu et *al.* (2012) reported the removal of 0.81% of the 625,875 records as errors or outliers but noted the high accuracy of information provided by the database. To provide an overview of insecticide use related to *A. transitella* control, I used single pooled values for each selected insecticide each year as opposed to the hundreds or thousands of individual applications, which may have led to incorporation of some record errors.

RESULTS

Sequencing and bioassays

Sanger sequencing the *para* locus confirmed that the point mutation conferring resistance to pyrethroids was absent in all ten sequenced larvae from the SPIRL-1966 and CPQ laboratory strains. The point mutation was present in all 20 larvae sequenced from the CHICO strain

(Figure 3.2). Bioassays demonstrated that the *kdr* mutation in the CHICO, ALM, and R347 strains confers resistance to bifenthrin and DDT because the LC_{50} values for both insecticides were greater in all populations with the mutation compared to CPQ, which did not have the mutation. Median-lethal concentrations values were significantly greater in the CHICO (bifenthrin LC_{50}: 29.95 ppm (26.77 – 33.34)), ALM (bifenthrin LC_{50}: 7.45 ppm (5.90 – 9.64; DDT LC_{50}: 259.85 ppm (216.71 – 326.0)) , and R347 (bifenthrin LC_{50}: 24.77 ppm (18.19 – 33.09); DDT LC_{50}: 310.33 ppm (249.37 – 424.29)) than in the CPQ (DDT LC_{50}: 25.32 ppm (19.73 – 30.84) strain for both bifenthrin and DDT (Table 1). The LC_{50} values for the CHICO and R347 strains were significantly different from the ALM strain but not from each other. LC_{50} values for DDT did not differ between the ALM and the R347 strains, both displaying > 10-fold resistance to DDT compared to CPQ.

Insecticide use in almonds from 1990 to 2016 and annual crop value from 1995 to 2017

Prior to the introduction of methoxyfenozide in 2005, insecticide applications in almonds consisted mostly of organophosphates and pyrethroids (Figure 3.3, Table 3.2). Organophosphates, dominated by azinphos-methyl, chlorpyrifos, diazinon, and phosmet from 1995 to 2002, showed a steady decline in pounds applied and acres treated, although they were still the most heavily applied insecticide class in orchards based on pounds applied and treated acres. From 2002 to 2017, the pyrethroids displaced the organophosphates as the most widely applied insecticide class. The number of acres treated by pyrethroids increased by 22-fold from 1990 to 2009. In the eight years following 2009, the number of acres treated by pyrethroids rose an additional 2.6-fold, or 57-fold compared to 1990. Methoxyfenozide, the active ingredient of Intrepid® and Intrepid-Edge®, increased 5.9-fold in pounds applied from 2009 to 2017 and

surpassed the pyrethroids in this measure from 2013 to 2017. The diamide insecticides chlorantraniliprole and flubendiamide increased 3.9-fold in pounds applied from 2009 to 2017. The increase in number of acres treated by the diamides and methoxyfenozide from 2009 to 2016 were nearly identical at 4.6 and 4.5-fold increases, respectively. Although used for navel orangeworm control since 1997, the number of acres treated by spinosyn insecticides were relatively low compared to acreages treated with pyrethroids and organophosphates. Spinosad was the only insecticide belonging to this class until spinetoram applications began in 2007. Applications shifted as spinetoram became the more heavily applied representative, due in part to its pairing with methoxyfenozide as Intrepid-Edge®. Spinosyn insecticides as a class, represented predominantly by spinetoram following 2007, rose 8.1-fold in treated acres from 2013 to 2017.

The almond crop reached its highest value in 2013 and 2014 (Figure 3.4). During these two years, almonds were valued at $3.21 per pound and $4 per pound, respectively (NASS 2013, 2014). From 2009 to 2014, the total value of almonds rose from $2.33 billion to $7.48 billion, a 3.2-fold increase. The number of acres treated by insecticides during this period rose by 1.8-fold for the pyrethroids, 3.9-fold for methoxyfenozide, 3.8-fold for the diamides, and 7.9-fold for the spinosyns.

Insecticide use in pistachios from 1990 to 2017

In pistachio orchards, organophosphates were the insecticide class with the highest number of pounds applied from 1995 to 2006, but the use of pesticides in this class decreased sharply from 2006 to 2007 (Figure 3.5). From 2006 to 2007, organophosphates declined 3.9-fold in the pounds applied, due to 4.5-fold reductions in the number of pounds and 4.2-fold reductions

in the number of acres treated by phosmet. After 2007, pyrethroids surpassed the organophosphates and became the most heavily applied insecticide class for both pounds applied and treated acres (Figure 3.5, Table 3.3). Since 1995, growers have treated a greatest number of acres every year with pyrethroid insecticides. From 1995 to 2016, pyrethroid insecticides increased 9-fold by pounds applied and 32.7-fold by acres treated. The largest increase in pyrethroid applications in a single year occurred in 2011 to 2012, when pounds of active ingredient and treated acres rose 1.5-fold and 1.3-fold, respectively and in 2015 to 2016, when treated pound applied and treated acres both rose 1.3-fold. In general, applications of the newer chemistries, including the spinosyns, methoxyfenozide, and the diamides, did not start increasing until after 2012. From 2012 to 2017, methoxyfenozide applications increased 4-fold by pounds applied and 4.1-fold by treated acres. During the same time period, the diamide insecticides rose 3.7-fold by pounds applied and 3.6-fold by treated acres. In 2017, however, pyrethroid applications were still 1.9-fold greater by pounds applied and 5.7-fold greater for acres treated relative to methoxyfenozide, and 5.6-fold greater by pounds applied and 4.2-fold greater by acres treated than the diamide insecticides.

Total pyrethroid use compared to bifenthrin use in almond and pistachio orchards in the year 2010

Tables 3.4 and 3.5 compare total pyrethroid use across all counties where *A. transitella* populations were collected. The ratio of pounds applied to treated acres, measured by *UI* were consistent across each year examined, ranging from 0.09-0.12 in statewide data. *UI* values were generally similar for Kern, Madera, and Colusa-Yolo Counties from 2000 to 2017. One exception occurred from 2011 to 2012 in Madera County, where the *UI* rose because pounds of

pyrethroids increased 1.8-fold while treated acreage only increased 1.1-fold. In pistachio orchards, the *UI* values were consistently above 0.2 from 2000 to 2009 but declined from 2010 to 2017 for statewide, Kern, and Madera County pyrethroid applications. In Kern and Madera pistachio orchards, the largest increase in pyrethroid applications in a single year occurred from 2011 to 2012, when pounds of active ingredient and treated acres rose 1.5-fold and 1.3-fold, respectively for Kern County and 1.4-fold and 1.1-fold in Madera County. Pyrethroid sprays in Colusa and Yolo Counties were consistently lower than Kern County and Madera County every year from 2000 to 2016 in almond orchards for total applications, pounds applied, and treated acres. Pistachio applications were not tracked for Colusa-Yolo Counties because 96% of production occurs in the southern San Joaquin Valley (Geissler and Horwath 2016).

Bifenthrin use in almond and pistachio orchards

Table 3.6 tracks bifenthrin applications in almond and pistachio orchards in Kern County, Madera County, and statewide since registration occurred in 2006 in almonds and 2008 in pistachios. Bifenthrin use increased heavily from 2009 to 2013 before resistance was first reported in Kern County almond orchards. Statewide use of bifenthrin increased 5.2-fold by pounds applied and 3.5-fold by treated acres from 2009 to 2013. Kern County applications in almonds increased 2.9-fold by pounds of bifenthrin applied and 2.1-fold by treated acres, while Madera County applications increased 9.9-fold in pounds applied and 5.8-fold by treated acres (Table 3.6). Brigade® WSB was the only registered product with bifenthrin as the active ingredient in almonds from 2006 to 2009 (Table 3.7).

Applications of bifenthrin increased in almonds as more products became registered for use. From 2010 to 2013, 13 products containing bifenthrin were applied in almond orchards. The

number of registered products continued to increase, and, in 2017, 19 products containing bifenthrin were used to treat almond orchards. When compared against the total pyrethroid use in almonds, bifenthrin applications represented at least 70% of total pyrethroid use by pounds of active ingredient throughout California every year from 2011 to 2017, except for 2011 (65.1%) and 2013 (69.8%) (Figure 3.6). From 2011 to 2017, bifenthrin represented 70.9% of all pounds of pyrethroids applied on average in Kern County and 75.1% of all pyrethroids applied on average in Madera County (Figure 3.7).

Statewide, Kern, and Madera County bifenthrin use in pistachio orchards rose by 5.4-, 3.6-, and 4-fold, respectively for pounds applied and 4.2-, 3.3-, and 2.3-fold by treated acres from 2009 to 2013. Bifenthrin applications in pistachio orchards represented on average 51.1% of all pounds of pyrethoids applied statewide, 59.6% applied in Kern County, and 45.8% applied in Madera County from 2011 to 2017 (Figure 3.8). Inspection of product use revealed that Brigade® WSB was the only registered insecticide containing bifenthrin from 2008 to 2009, but 9 insecticides containing bifenthrin were registered and applied from 2010 to 2013 (Table 3.8). By 2017, 16 products containing bifenthrin were applied in pistachio orchards.

DISCUSSION

The high economic returns of California almonds and pistachios have resulted in substantial increases in the use of insecticides in order to reduce damage and maximize profits for growers. This review of insecticide use supported the analyses on pesticide use in almonds and pistachio orchards performed by Van Steenwyk *et al.* (2017) from 2000 to 2014. However, my approach was more focused on selected insecticides applied most frequently for *A. transitella* management and documents the shift away from the organophosphates and toward pyrethroids as

the primary insecticides. Pyrethroids have been used for management in almond and pistachio orchards of many pests other than *A. transitella*, including small plant bugs (*Calochoris norvegicus, Phytocoris relativus, Phytocoris californicus*), leaf-footed bugs (*Leptoglossus clypealis, Leptoglossus zonatus, Leptoglossus occidentalis*), and stink bugs (*Chlorochroa uhleri, Acrosternum hilare, Thyanta pallidovirens*) (Demkovich *et al.* 2015a, Joseph and Bolda 2016, Van Steenwyk *et al.* 2017). Consequently, this discussion of insecticide records based on county averages for a single pest is limited to a discussion of general trends.

Bifenthrin became the dominant representative of pyrethroid insecticides applied in almonds orchards by 2011 and pistachio orchards by 2013. Growers favored bifenthrin because they liked the adult and egg kill for *A. transitella*, and the fact that it did not flare spider mites (*Tetranychus pacificus, Tetranychus* urticae) in almond orchards, unlike most pyrethroids (Zalom *et al.*, 2001, Hamby *et al.*, 2013). Another factor likely driving the surge in applications, especially from 2010 to 2013, was cost. Brigade® went off patent in 2009 (J. Siegel, personal communication), facilitating the emergence of generics with bifenthrin as the active ingredient (Tables 3.7, 3.8). After chemical patents expire, competing companies typically produce generic forms of the previously patented chemicals at dramatically reduced costs (Sexton *et al.* 2007)**.** When Brigade® WSB was registered for application in almonds in 2006, treating an acre with bifenthrin cost >22 dollars and by 2012 the cost had declined to 4 to 5 dollars to treat an acre using the new generic Bifenture 2 EC® (J. Siegel, personal communication). The expiration of the bifenthrin patent under Brigade® in almonds and pistachios caused a decline in product use and shift towards newly registered products such as Fanfare 2 EC®, Bifenture 2 EC®, and Sniper®. There were more pounds of each of these bifenthrin products applied from 2011 to 2013 in almond and pistachio orchards relative to Brigade®. The *UI* measures during this time period

were greater for Fanfare®, Bifenture®, and Sniper®, and *A. transitella* may have faced elevated selection pressure in orchards that were applying these other formulations of bifenthrin.

The interface between almonds and pistachios grown in the Central Valley of California can often complicate insecticide management strategies for growers. Analysis of the insecticide records shows that similar almond and pistachio acreages were treated with pyrethroids. Because there can be three to four generations of *A. transitella* in the Central Valley, populations likely received elevated pyrethroid exposure in orchards of both nut crops during overlapping periods of susceptibility. Although insecticide applications increased at higher rates in Madera County than for Kern County following the patent expiration of Brigade®, resistance was first reported in Kern County almond orchards in 2013 (Demkovich *et al.* 2015a). This insecticide use pattern reported supports the hypothesis that there was greater bifenthrin and total pyrethroid selection pressure in Kern County pistachio orchards than in Madera County orchards, which may have contributed to initial resistance development in Kern County. However, because the records analyzed here reflect pooled data, I could not analyze site-specific differences in application, which is important for Kern County. In this county, fewer than ten companies controlled 75 percent of the almond acreage. Spray decisions may be influenced by managers trying to reduce rejected nuts at any cost as part of their job performance (Goodhue 2010), and the management decisions made by these few companies may have had a disproportionate effect on selection for resistance. Because my analytic approach is based strictly on pooled averages, I had no information on site-specific differences in application, which may have heavily influenced resistance outbreaks.

In order to determine if resistance had spread beyond the San Joaquin Valley and into the Sacramento Valley, I sequenced the *para* gene in the CHICO strain from Colusa and Yolo

Counties and found that all 20 larvae sequenced had the point mutation described in Calla *et al.* (in preparation) that confers resistance to pyrethroids. Using feeding assays, I determined that the median-lethal concentration for the CHICO strain was greater than the ALM strain from Madera County but not different from the pyrethroid-resistant R347 strain from Kern County. I also carried out feeding assays using DDT with the ALM and R347 strains examined earlier (Chapter 1) and found that the presence of the *kdr* mutation also confers resistance to DDT. The differences observed in the LC_{50} values from these bioassays among the ALM, CHICO, and R347 strains relative to the CPQ laboratory strain follow the pattern observed for the sequencing results, with those strains possessing the *kdr* mutation being more resistant to DDT.

The presence of the point mutation in the CHICO strain appears to be inconsistent with the insecticide use records. Pistachios are essentially restricted to the San Joaquin Valley, and records of insecticide use in almonds show that pyrethroid application measures did not increase to levels comparable to those in Kern and Madera Counties until 2015 and 2016. Additional information about pesticide usage in 2018 and 2019 in almonds may provide more insight as to the magnitude of the selection pressure from pyrethroid use in this region. It is also possible that pyrethroid use to protect walnuts and stone fruit contributed to the selection pressure in this region (CDPR 2017).

Regardless, sequencing the target *para* gene for the *kdr* mutation and confirming its presence through effective bioassays can be used as an assessment tool for tracking a common pyrethroid resistance mechanism. In turn, a better understanding of the distribution of this mutation throughout the Central Valley will help managers devise a more effective strategy to conserve pyrethroids. Records of pesticide use available through the California DPR provide the history of applications in a given region and serve as a useful tool for understanding application

strategies of all insecticide classes adopted by growers. These records can also offer an understanding of how insecticide use may facilitate resistance evolution in a given area, and, when combined with genetic surveys for resistance mutations, can offer greater insight into the distributions of resistant populations.

TABLES AND FIGURES

Table 3.1. Median-lethal concentration results obtained through first instar feeding assays on artificial diets with the ALM, CHICO, CPQ, and R347 strains for bifenthrin and DDT.

Population	Bifenthrin 48 h LC50 (95% CI)	DDT 48 h LC50 (95% CI)	*kdr* Mutation
ALM	7.45 ppm (5.90 - 9.64)*	259.85 ppm (216.71 - 326.0)	yes
CHICO	29.95 ppm (26.77 - 33.34)	————	yes
R347	24.27 ppm (18.19 - 33.09)*	310.33 ppm (249.37 - 424.29)	yes
CPQ	0.38 ppm (0.31 - 0.46)**	25.32 ppm (19.73 - 30.84)	no

* Median-lethal concentrations obtained from Chapter 1 experiments with ALM and R347 populations

** Median-lethal concentration obtained from Bagchi *et al.* (2016)

Table 3.2. Statewide insecticide use in almond orchards for the pyrethroids (bifenthrin, cyfluthrin, beta-cyfluthrin, (S) – cypermethrin, esfenvalerate, fenpropathrin, gamma-cyhalothrin, lambda-cyhalothrin, permethrin), methoxyfenozide, spinosyns (spinetoram, spinosad) and diamides (chlorantraniliprole, flubendiamide) from 2000 – 2017. *UI* = pounds applied / treated acres.

Almond Use	Pyrethroids				Methoxyfenozide				Spinosyns				Diamides			
	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2000	3,138	24,234	216,486	0.11	––	––	––	––	628	3,862.94	42,486.18	0.09	––	––	––	––
2001	3,438	27,991	210,969	0.13	––	––	––	––	260	1,812.62	19,691.76	0.09	––	––	––	––
2002	3,558	28,745	239,179	0.12	––	––	––	––	215	1,017.77	12,451.80	0.08	––	––	––	––
2003	3,723	26,697	242,057	0.11	––	––	––	––	263	1,512.88	15,422.02	0.10	––	––	––	––
2004	4,765	26,989	298,886	0.09	913	15,021	61,268	0.25	305	1,449.10	18,492.01	0.08	––	––	––	––
2005	6,490	43,728	459,610	0.10	1,883	36,964	144,918	0.26	452	3,410.96	39,954.75	0.09	––	––	––	––
2006	6,413	47,148	461,652	0.10	2,441	50,144	192,689	0.26	404	3,039	34,256	0.09	––	––	––	––
2007	7,075	50,968	463,626	0.11	2,780	55,742	216,235	0.26	445	2,643	28,351	0.09	––	––	––	––
2008	7,232	42,375	492,431	0.09	2,785	61,358	235,077	0.26	340	1,777	24,332	0.07	––	––	––	––
2009	6,490	43,866	463,723	0.09	1,883	36,964	144,918	0.26	202	937	12,231	0.08	1,873	14,424	144,212	0.10
2010	8,476	70,936	627,651	0.11	2,583	53,746	208,000	0.26	146	439	6,014	0.07	2,029	14,532	151,336	0.10
2011	9,321	84,057	710,871	0.12	2,621	45,776	180,066	0.25	265	1,203	16,681	0.07	2,394	16,817	179,778	0.09
2012	9,740	87,365	734,539	0.12	3,319	58,607	242,574	0.24	222	1,138	15,181	0.07	3,169	21,238	230,938	0.09
2013	13,189	102,950	963,662	0.11	5,651	125,533	448,808	0.28	442	2,943	32,140	0.09	5,805	42,463	448,858	0.09
2014	11,634	90,865	817,997	0.11	7,330	160,411	559,294	0.29	1,461	7,635	96,920	0.08	7,241	53,791	553,495	0.10
2015	16,264	127,702	1,158,703	0.11	8,674	191,070	661,728	0.29	3,722	17,095	254,991	0.07	8,484	65,505	664,044	0.10
2016	15,083	113,576	1,023,707	0.11	9,510	193,544	658,307	0.29	4,296	18,575	272,370	0.07	8,470	65,128	666,360	0.10
2017	17,734	131,633	1,192,047	0.11	9,510	193,544	658,307	0.29	4,296	18,575	272,370	0.07	8,470	65,128	666,360	0.10

Table 3.3. Statewide insecticide use in pistachio orchards for the pyrethroids (bifenthrin, cyfluthrin, beta-cyfluthrin, (S) – cypermethrin, fenpropathrin, lambda-cyhalothrin, permethrin), methoxyfenozide, spinosyns (spinetoram, spinosad) and diamides (chlorantraniliprole, flubendiamide) from 2000 – 2017. *UI* = pounds applied / treated acres.

Pistachio Use	Pyrethroids				Methoxyfenozide				Spinosyns				Diamides			
	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2000	1,252	26,778	108,397	0.25	––	––	––	––	33	284	3,257	0.09	––	––	––	––
2001	764	15,972	67,032	0.24	––	––	––	––	36	1,248	5,622	0.22	––	––	––	––
2002	956	22,862	91,318	0.25	––	––	––	––	29	384	4,177	0.09	––	––	––	––
2003	1,085	31,650	116,337	0.27	––	––	––	––	18	210	2,859	0.07	––	––	––	––
2004	1,601	39,081	146,901	0.27	139	3,312	13,580	0.24	18	470	7,128	0.07	––	––	––	––
2005	1,786	48,424	172,797	0.28	250	8,060	27,381	0.29	13	178	1,883	0.09	––	––	––	––
2006	2,563	66,363	229,382	0.29	523	15,620	54,843	0.28	22	393	4,951	0.08	––	––	––	––
2007	2,173	59,980	220,186	0.27	418	16,569	54,513	0.30	6	69	1,218	0.06	––	––	––	––
2008	1,907	41,671	199,460	0.21	427	11,553	38,142	0.30	6	48	460	0.11	––	––	––	––
2009	2,532	54,995	265,433	0.21	369	14,250	46,192	0.31	11	103	1,098	0.09	91	1,914	8,248	0.23
2010	3,154	48,375	311,133	0.16	388	14,290	43,223	0.33	14	263	2,159	0.12	161	943	12,068	0.08
2011	4,098	48,569	408,501	0.12	450	16,882	46,950	0.36	14	112	1,148	0.10	360	2,072	25,810	0.08
2012	5,610	71,027	522,332	0.14	362	13,547	37,321	0.36	43	363	4,262	0.09	753	5,021	58,049	0.09
2013	6,219	80,227	602,634	0.13	824	37,078	95,229	0.39	110	1,238	11,956	0.10	1,299	11,639	124,089	0.09
2014	6,740	72,101	612,700	0.12	1,199	47,187	129,036	0.37	340	2,633	30,031	0.09	1,698	17,787	183,438	0.10
2015	6,981	80,628	632,868	0.13	1,226	46,028	122,540	0.38	640	3,723	51,874	0.07	1,681	16,385	166,631	0.10
2016	8,054	105,009	816,897	0.13	1,142	47,270	125,371	0.38	688	3,231	46,304	0.07	1,861	18,126	186,160	0.10
2017	8,662	103,575	873,320	0.12	1,544	54,650	153,077	0.36	749	4,496	66,951	0.07	2,064	18,354	208,388	0.09

Table 3.4. Total pyrethroid use (bifenthrin, cyfluthrin, beta-cyfluthrin, (S) – cypermethrin, esfenvalerate, fenpropathrin, gamma-cyhalothrin, lambda-cyhalothrin, permethrin) in almond orchards for Kern County, Madera County, Colusa-Yolo Counties, and across all of California from 2000 – 2016. *UI* = pounds applied / treated acres.

Pyrethroid Use in	**Statewide**				**Kern County**				**Madera County**				**Colusa-Yolo Counties**			
Almonds	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2000	3,138	24,234	216,486	0.11	525	6,393	58,788	0.11	170	2,764	17,651	0.16	38	170	3,686	0.05
2001	3,438	27,991	210,969	0.13	405	7,910	41,606	0.19	252	3,464	23,458	0.15	70	210	3,865	0.05
2002	3,558	28,745	239,179	0.12	415	5,883	40,410	0.15	311	4,298	28,499	0.15	43	142	3,629	0.04
2003	3,723	26,697	242,057	0.11	358	3,077	32,019	0.10	354	4,587	31,507	0.15	32	131	2,835	0.05
2004	4,765	26,989	298,886	0.09	615	4,821	54,517	0.09	489	4,003	36,559	0.11	25	113	1,890	0.06
2005	6,490	43,728	459,610	0.10	487	3,733	42,186	0.09	438	4,588	32,599	0.14	68	276	3,515	0.08
2006	6,413	47,148	461,652	0.10	713	6,625	59,611	0.11	665	6,678	54,626	0.12	163	858	11,973	0.07
2007	7,075	50,968	463,626	0.11	787	6,976	70,304	0.10	719	6,166	48,886	0.13	175	664	6,319	0.11
2008	7,232	42,375	492,431	0.09	955	8,912	88,980	0.10	612	2,824	42,357	0.07	365	1,025	16,836	0.06
2009	6,490	43,866	463,723	0.09	988	10,266	96,799	0.11	581	4,680	39,349	0.12	333	1,413	17,352	0.08
2010	8,476	70,936	627,651	0.11	1,274	16,866	138,748	0.12	1,082	8,515	76,183	0.11	284	1,133	10,881	0.10
2011	9,321	84,057	710,871	0.12	1,696	19,989	185,568	0.11	1,047	11,311	80,011	0.14	406	1,921	18,339	0.10
2012	9,740	87,365	734,539	0.12	1,561	21,384	183,468	0.12	1,292	19,958	95,505	0.21	384	2,105	23,136	0.09
2013	13,189	102,950	963,662	0.11	1,671	22,460	181,507	0.12	2,047	14,504	139,317	0.10	713	3,807	40,285	0.09
2014	11,634	90,865	817,997	0.11	1,217	12,522	111,544	0.11	1,783	15,589	121,970	0.13	726	4,338	40,317	0.11
2015	16,264	127,702	1,158,703	0.11	1,754	21,527	195,674	0.11	2,363	21,948	171,138	0.13	1,159	5,956	59,211	0.10
2016	15,083	113,576	1,023,707	0.11	1,563	16,465	150,853	0.11	1,884	17,187	132,858	0.13	1,072	5,849	59,915	0.10
2017	17,734	131,633	1,192,047	0.11	1,552	19,382	148,608	0.13	2,175	18,394	148,552	0.12	1,324	7,776	86,850	0.09

Table 3.5. Total pyrethroid use (bifenthrin, cyfluthrin, beta-cyfluthrin, (S) – cypermethrin, fenpropathrin, lambda-cyhalothrin, permethrin) in pistachio orchards for Kern County, Madera and across all of California from 2000 – 2017. *UI* = pounds applied / treated acres.

Pyrethroid Use	**Statewide**				**Kern County**				**Madera County**			
in Pistachios	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2000	1,252	26,778	108,397	0.25	192	8,823	38,117	0.23	684	9,320	37,745	0.25
2001	764	15,972	67,032	0.24	101	4,180	21,880	0.19	424	6,303	24,217	0.26
2002	956	22,862	91,318	0.25	235	8,370	35,918	0.23	481	7,990	29,503	0.27
2003	1,085	31,650	116,337	0.27	300	15,884	56,991	0.28	431	6,104	23,108	0.26
2004	1,601	39,081	146,901	0.27	335	12,284	50,153	0.24	735	12,640	44,763	0.28
2005	1,786	48,424	172,797	0.28	569	19,307	72,376	0.27	562	11,063	39,533	0.28
2006	2,563	66,363	229,382	0.29	581	20,569	82,579	0.25	887	17,813	56,078	0.32
2007	2,173	59,980	220,186	0.27	627	26,305	97,034	0.27	729	12,847	49,731	0.26
2008	1,907	41,671	199,460	0.21	657	19,002	90,213	0.21	386	5,691	28,864	0.2
2009	2,532	54,995	265,433	0.21	756	25,136	113,319	0.22	724	6,950	53,589	0.13
2010	3,154	48,375	311,133	0.16	770	21,386	110,445	0.19	964	8,932	69,594	0.13
2011	4,098	48,569	408,501	0.12	1,040	16,283	144,843	0.11	1,197	9,514	86,717	0.11
2012	5,610	71,027	522,332	0.14	1,204	22,460	162,571	0.14	2,071	15,750	127,867	0.12
2013	6,219	80,227	602,634	0.13	1,416	26,194	181,256	0.14	1,648	13,647	109,095	0.13
2014	6,740	72,101	612,700	0.12	1,289	17,498	146,692	0.12	1,839	14,796	126,331	0.12
2015	6,981	80,628	632,868	0.13	1,433	20,995	169,886	0.12	1,497	13,709	117,694	0.12
2016	8,054	105,009	816,897	0.13	1,699	28,359	212,310	0.13	1,631	16,956	127,407	0.13
2017	8,662	103,575	873,320	0.12	1,806	27,136	225,641	0.12	1,636	16,171	134,680	0.12

Table 3.6. Bifenthrin use in almond and pistachio orchards in Kern County, Madera County, Colusa-Yolo Counties, and statewide. Pistachio applications were omitted for Colusa-Yolo Counties because the crop is not grown in that area.

Bifenthrin Use	**Statewide Almonds**				**Kern County Almonds**				**Madera County Almonds**				**Colusa-Yolo County Almonds**			
	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2006	433	3,904	32,456	0.12	22	294	2,081	0.14	42	239	2,528	0.09	59	352	2,633	0.13
2007	1,401	9,844	96,955	0.10	225	2,720	21,839	0.12	189	921	9,898	0.09	107	398	5,290	0.08
2008	1,297	10,086	99,982	0.10	238	3,354	26,884	0.12	138	769	8,183	0.09	150	471	5,810	0.08
2009	1,407	13,775	123,524	0.11	369	5,824	44,840	0.13	156	942	8,888	0.11	234	988	10,910	0.09
2010	2,235	28,510	205,436	0.14	533	10,166	64,024	0.16	344	3,191	22,938	0.14	186	918	8,601	0.11
2011	3,198	54,683	270,259	0.20	660	12,656	79,746	0.16	394	7,542	29,536	0.26	212	1,571	12,060	0.13
2012	4,302	62,369	359,767	0.17	764	14,412	88,953	0.16	670	16,331	52,559	0.31	196	1,706	13,065	0.13
2013	5,606	71,854	434,400	0.17	792	16,694	95,188	0.18	808	9,320	51,815	0.18	356	2,559	18,895	0.14
2014	5,802	68,716	432,712	0.16	465	8,971	51,112	0.18	1,015	12,345	69,283	0.18	512	3,788	29,177	0.13
2015	7,606	93,718	569,197	0.16	666	14,966	83,831	0.18	1,312	17,919	96,956	0.18	652	1,281	8,652	0.15
2016	6,916	81,904	495,528	0.17	579	11,552	64,492	0.18	995	12,907	69,133	0.19	547	4,148	30,882	0.13
2017	7,917	96,161	577,750	0.17	829	15,701	86,328	0.18	1,045	14,167	79,277	0.18	699	5,312	46,892	0.11
	Statewide Pistachios				**Kern County Pistachios**				**Madera County Pistachios**							
	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*				
2008	230	4,605	29,214	0.16	101	2,764	16,075	0.17	32	268	2,637	0.10				
2009	455	7,746	53,839	0.14	182	4,654	26,606	0.17	142	1,353	13,047	0.10				
2010	463	7,903	53,849	0.15	160	4,400	25,722	0.17	68	548	5,626	0.10				
2011	946	18,646	111,602	0.17	320	9,728	53,532	0.18	271	3,080	21,392	0.14				
2012	1,600	32,109	174,014	0.18	479	14,234	69,026	0.21	482	6,191	35,061	0.18				
2013	2,183	42,078	231,912	0.18	599	16,942	88,311	0.19	493	5,474	30,031	0.18				
2014	2,426	41,397	227,620	0.18	447	10,333	53,881	0.19	660	8,196	46,522	0.18				
2015	2,345	41,713	228,884	0.18	425	10,790	56,106	0.19	501	7,358	40,157	0.18				
2016	3,276	61,154	343,590	0.18	680	16,465	89,100	0.18	658	8,721	47,191	0.18				
2017	2,978	56,154	312,054	0.18	734	16,517	94,205	0.18	503	7,843	41,046	0.19				

Table 3.7. Bifenthrin use as the active ingredient(s) under registered trade names from 2006 – 2017 in almond orchards. Usage intensity (*UI*) is equal to the pounds of bifenthrin applied for each product divided by the treated acres. Trade names which comprise "Other" include Bifenture® EC-CA, Capture® EC-Cal, Swagger®, Helena Bifenthrin® 2EC-Cal, Sniper® Helios, Bifen 2 Ag Gold-Cal, Brigade® 2EC, Fanfare® EC, SPECKoZ® Bifenthrin, Bifenture® LFC, and Bifen 25% EC.

Statewide	Brigade® WSB				Fanfare™ 2EC				Bifenture® 10DF				Bifenture® EC			
Almond Use	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*
2006	434	3,904	32,456	0.12	––	––	––	––	––	––	––	––	––	––	––	––
2007	1,398	9,979	96,946	0.10	––	––	––	––	––	––	––	––	––	––	––	––
2008	1,310	10,403	103,107	0.10	––	––	––	––	––	––	––	––	––	––	––	––
2009	1,433	13,819	123,986	0.11	––	––	––	––	––	––	––	––	––	––	––	––
2010	1,101	10,364	91,170	0.11	836	16,112	93,979	0.17	300	2,035	20,287	0.10	––	––	––	––
2011	683	5,574	51,574	0.11	743	12,904	74,310	0.17	139	737	9,530	0.08	1,326	29,504	112,093	0.26
2012	709	5,852	52,406	0.11	925	15,900	90,989	0.17	113	1,061	9,865	0.11	2,023	34,263	170,186	0.20
2013	772	6,144	52,306	0.12	516	6,207	33,981	0.18	127	1,104	10,995	0.10	3,167	41,515	242,981	0.17
2014	673	4,728	42,858	0.11	80	1,016	4,146	0.25	67	449	4,216	0.11	2,795	38,500	230,815	0.17
2015	799	5,794	51,726	0.11	99	518	3,322	0.16	90	499	4,729	0.11	3,437	50,974	281,786	0.18
2016	618	4,633	41,363	0.11	165	1,660	8,651	0.19	38	256	1,721	0.15	2,771	39,664	221,537	0.18
2017	622	4,364	48,724	0.09	183	1,763	10,428	0.17	8	60	547	0.11	2,950	34,773	202,369	0.172
	Sniper®				Athena®				Brigadier®				Hero® EW			
	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*
2011	140	5,044	12,840	0.39	158	901	9,603	0.09	1	7	65	0.10	3	13	243	0.053
2012	291	3,834	21,232	0.18	229	1,389	14,499	0.10	1	29	285	0.10	––	––	––	––
2013	604	9,226	49,684	0.19	188	1,490	12,872	0.12	9	56	702	0.08	1	3	40	0.075
2014	565	9,959	52,676	0.19	239	1,606	14,275	0.11	25	144	2,275	0.06	73	176	3,641	0.05
2015	1,026	17,569	92,445	0.19	311	2,074	18,394	0.11	109	424	5,554	0.08	96	397	6,898	0.06
2016	986	15,204	80,879	0.19	130	519	4,882	0.11	109	446	6,060	0.07	20	68	1,166	0.06
2017	1,293	25,618	132,966	0.193	121	586	5,300	0.11	28	32	611	0.05	92	264	4,441	0.06
	Bifen 2 Ag Gold				Fanfare® ES				Aceto Bifenthrin 2EC				Other			
	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*
2007	––	––	––	––	––	––	––	––	––	––	––	––	3	0.89	9.5	0.09
2008	––	––	––	––	––	––	––	––	––	––	––	––	2	0.55	5.5	0.10
2009	––	––	––	––	––	––	––	––	––	––	––	––	––	––	––	––
2010	––	––	––	––	––	––	––	––	––	––	––	––	––	––	––	––
2011	––	––	––	––	––	––	––	––	––	––	––	––	––	––	––	––
2012	––	––	––	––	––	––	––	––	––	––	––	––	3	42	305	
2013	––	––	––	––	325	4,978	25,036	0.20	––	––	––	––	38	1,132	5,805	0.20
2014	878	6,716	48,885	0.14	105	1,251	6,618	0.19	303	4,138	22,062	0.19	13	33	276	0.12
2015	919	6,430	49,713	0.13	246	3,260	17,368	0.19	432	5,139	32,214	0.16	47	628	4,820	0.13
2016	969	6,648	50,174	0.133	410	4,156	28,788	0.14	666	8,282	47,522	0.17	62	370	2,785	0.133
2017	963	7,251	49,020	0.148	616	7,110	40,214	0.18	772	11,092	64,190	0.17	302	3,227	18,939	0.17

Table 3.8. Bifenthrin use as the active ingredient(s) under registered trade names from 2008 to 2017 in pistachio orchards. Usage intensity (*UI*) is equal to the pounds of bifenthrin applied for each product divided by the treated acres. Trade names which comprise "Other" include Bifenture® EC-CA, Brigadier®, Hero EW®, Bifen 25% EC, and Fanfare® EC.

Statewide	Brigade® WSB				Fanfare™ 2EC				Bifenture® 10DF				Bifenture® EC			
Pistachio Use	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*	Applications	Pounds Bifenthrin	Treated Acres	*UI*
2008	230	4,605	29,214	0.16	––	––	––	––	––	––	––	––	––	––	––	––
2009	455	7,746	53,839	0.14	––	––	––	––	––	––	––	––	––	––	––	––
2010	**298**	**2,817**	**25,607**	**0.11**	**151**	**4,774**	**25,308**	**0.19**	**14**	**312**	**2,934**	**0.11**	––	––	––	––
2011	139	1,535	12,807	0.12	158	2,642	14,780	0.18	75	662	6,788	0.10	547	13,515	75,702	0.18
2012	247	3,173	23,692	0.13	187	3,824	18,355	0.21	225	2,384	15,742	0.15	728	17,802	95,688	0.19
2013	199	2,113	16,452	0.13	229	3,199	16,531	0.19	32	551	3,744	0.15	1,137	26,895	143,543	0.19
2014	194	1,550	14,309	0.11	62	741	4,125	0.18	42	603	4,106	0.15	1,509	28,000	147,542	0.19
2015	206	2,023	16,088	0.13	32	342	1,933	0.18	35	530	4,251	0.12	1,213	26,708	139,045	0.19
2016	163	1,900	13,488	0.14	75	1,630	8,025	0.20	98	1,020	7,786	0.13	1,544	36,497	198,596	0.18
2017	94	1,035	10,610	0.098	12	97	681	0.14	42	357	2,929	0.12	1,191	22,847	124,572	0.183
	Sniper®				**Fanfare® ES**				**Aceto Bifenthrin 2EC**				**Brigade® 2EC**			
	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2011	26	293	1,525	0.19	––	––	––	––	––	––	––	––	––	––	––	––
2012	216	4,873	20,278	0.24	1	22	110	0.20	––	––	––	––	––	––	––	––
2013	357	5,787	31,478	0.18	204	3,265	18,794	0.17	––	––	––	––	14	179	897	0.20
2014	330	5,703	29,972	0.19	44	498	2,759	0.18	176	3,361	18,824	0.18	4	167	888	0.188
2015	417	6,830	35,765	0.19	102	1,724	8,922	0.19	187	2,339	13,230	0.18	––	––	––	––
2016	718	11,267	64,526	0.17	275	3,541	19,706	0.18	257	3,734	20,491	0.18	5	35	187	0.187
2017	917	21,435	113,504	0.19	185	2,475	13,257	0.187	278	3,642	19,210	0.19	––	––	––	––
	Bifen 2 Ag Gold				**Sniper® Helios**				**Tempest™**				**Other**			
	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*	Applications	Pounds	Treated Acres	*UI*
2012	––	––	––	––	––	––	––	––	––	––	––	––	1	30	150	0.20
2013	––	––	––	––	––	––	––	––	––	––	––	––	8	89	472	0.19
2014	69	773	5,095	0.09	––	––	––	––	––	––	––	––	––	––	––	––
2015	113	850	5,220	0.16	––	––	––	––	46	367	4,430	0.08	––	––	––	––
2016	91	757	4,598	0.165	1	4	20	0.20	36	496	4,799	0.10	24	273	1,369	0.20
2017	112	1,225	7,460	0.164	50	418	2,166	0.19	14	73	818	0.09	78	2,416	16,186	0.15

Figure 3.1. Location of counties where *A. transitella* strains were collected. The CHICO strain was collected from Colusa and Yolo Counties. The ALM strain was collected from almond orchards in Madera County. R347 was collected from Kern County almond orchards at the same location where pyrethroid resistance was first described in 2013.

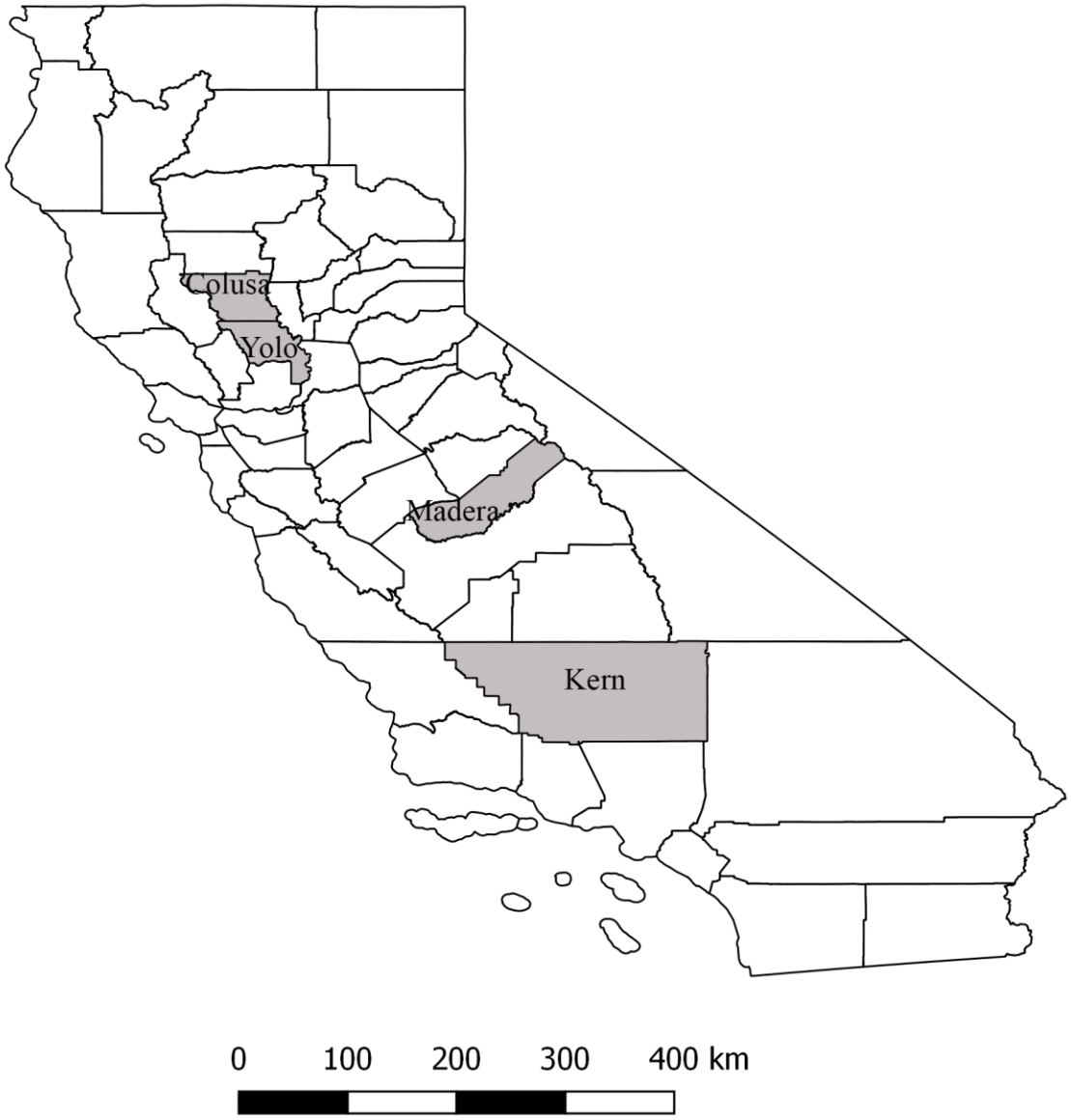


Figure 3.2. (A) 1.3 Mb selective sweep identified in Calla *et al.* (2019) by significantly reduced pi nucleotide diversity (Tajima's π) for a susceptible population collected from Madera County almond orchards (ALM), population from fig orchards in Madera County (FIG), and pyrethroid-resistant population from Kern County almond orchards (R347), with models provided for regions encoded by genes. **(B)** Examination of a single nucleotide substitution in the para gene present in ALM, FIG, and R347 populations which alters confirmation of the voltage gated sodium channel by changing a leucine to phenylalanine and confers resistance to pyrethroids. **(C)** Alignments of the para gene in an *A. transitella* population from Colusa and Yolo Counties (CHICO) and two laboratory strains CPQ and SPIRL-1966 (Reference genome strain). The red base T showcases the same substitution displayed in Figure 2B.

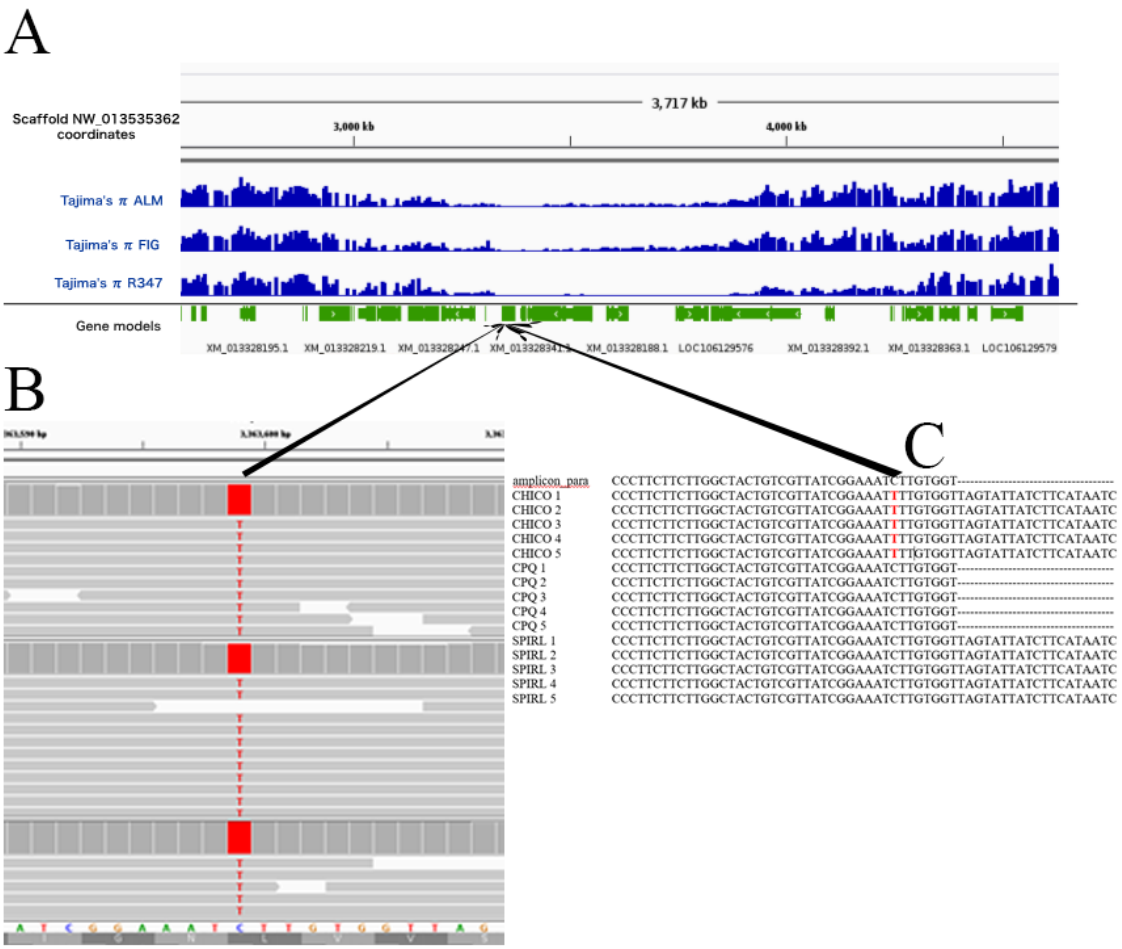


Figure 3.3. Insecticide applications for the organophosphates (azinphos-methyl, chlorpyrifos, diazonin, fenamiphos, malathion, methidathion, parathion, phosmet), pyrethroids (bifenthrin, cyfluthrin, beta-cyfluthrin, (S) – cypermethrin, esfenvalerate, fenpropathrin, lambda-cyhalothrin, permethrin), spinosyns (spinetoram, spinosad), methoxyfenozide, and the diamide insecticides (chlorantraniliprole, flubendiamide) from 1990 – 2017 in almond orchards.

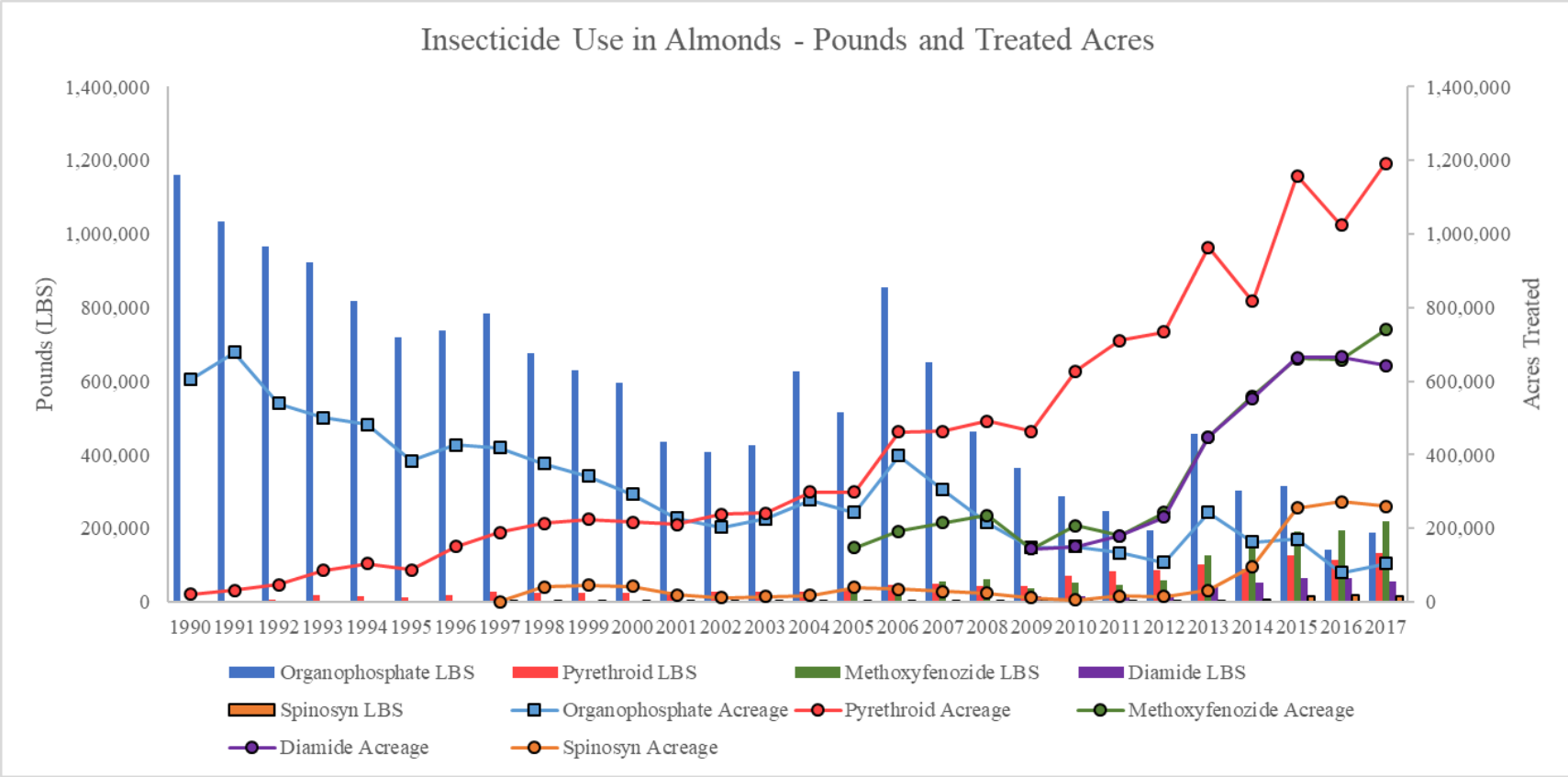


Figure 3.4. Almond crop value from 1995 to 2017 and acreage treated by insecticide classes used for *A. transitella* control. Almond crop values were calculated based on the annual price per pound and pounds produced. Almond data were obtained through California almond acreage reports provided by the United States Department of Agriculture National Agricultural Statistics Service (NASS).

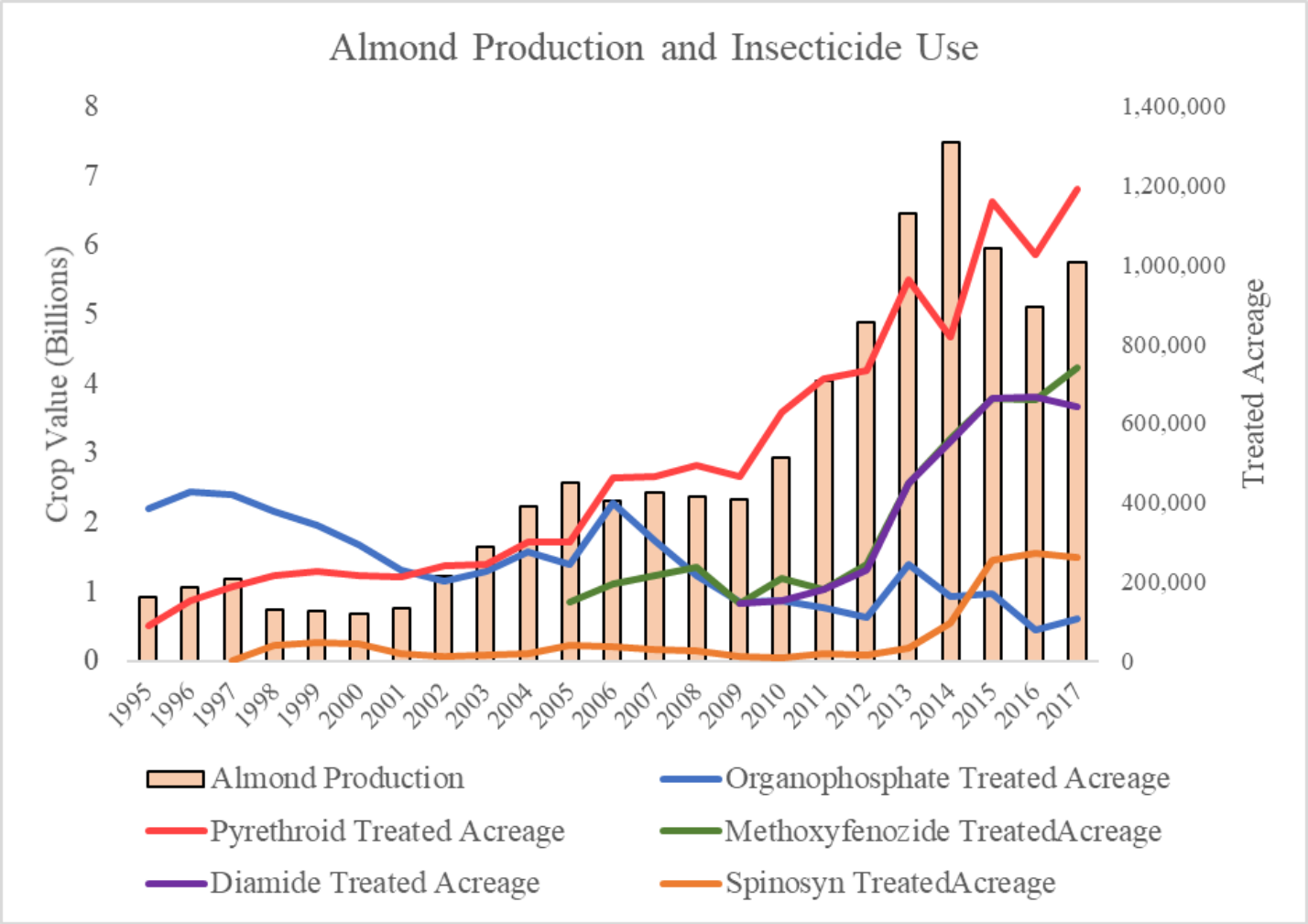


Figure 3.5. Insecticide applications for the organophosphates (azinphos-methyl, phosmet), pyrethroids (bifenthrin, cyfluthrin, beta-cyfluthrin, (S) – cypermethrin, fenpropathrin, lambda-cyhalothrin, permethrin), spinosyns (spinetoram, spinosad), methoxyfenozide, and the diamide insecticides (chlorantraniliprole, flubendiamide) from 1995 – 2016 in pistachio orchards.

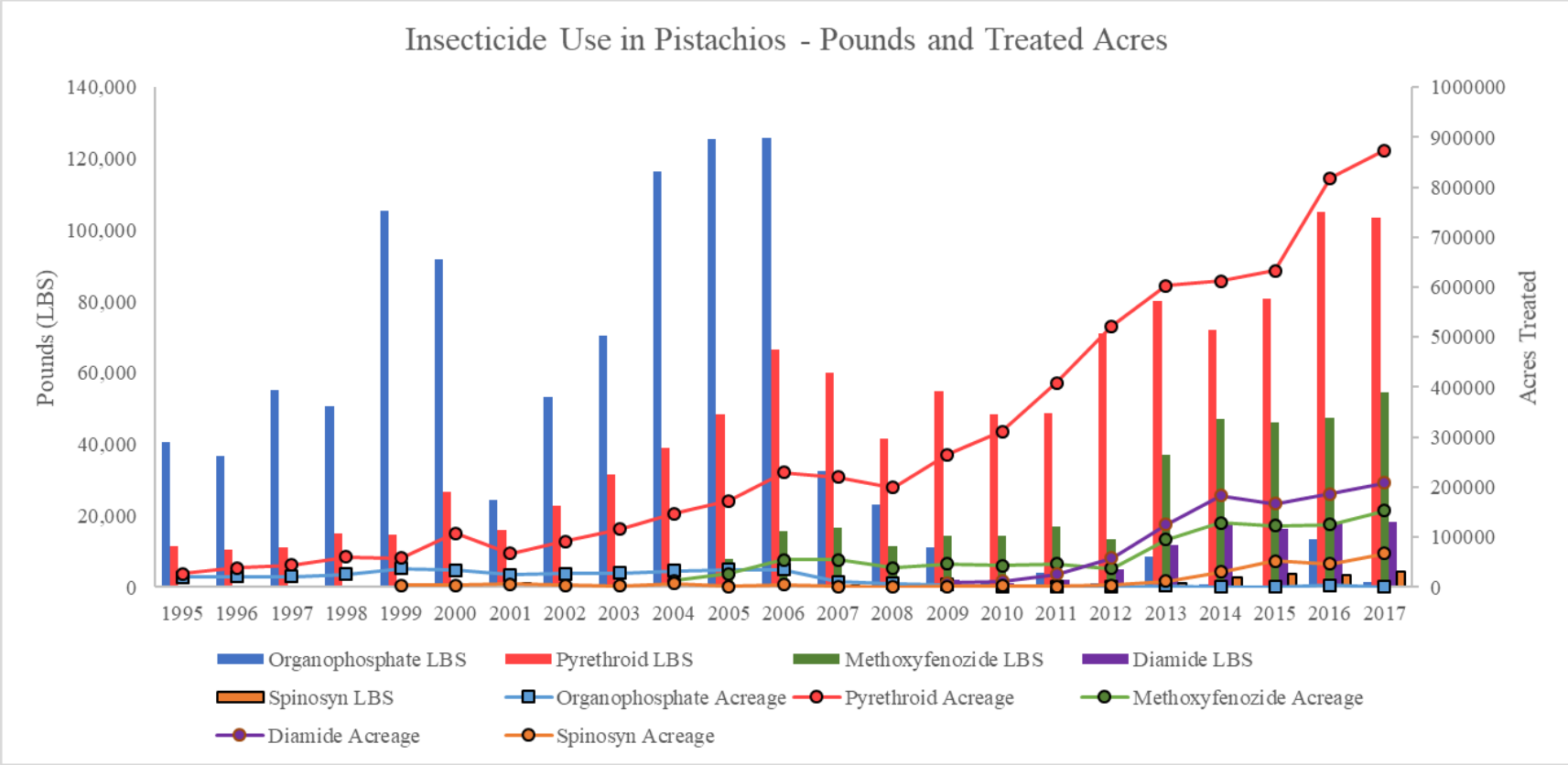


Figure 3.6. Bifenthrin use since its registration in 2006 versus all other pyrethroids reported in the DPR pesticide use records (cyfluthrin, beta-cyfluthrin, (S)-cypermethrin, deltamethrin, esfenvalerate, fenpropathrin, lambda-cyhalothrin, gamma-cyhalothrin, permethrin) in almond orchards from 2006 – 2017.

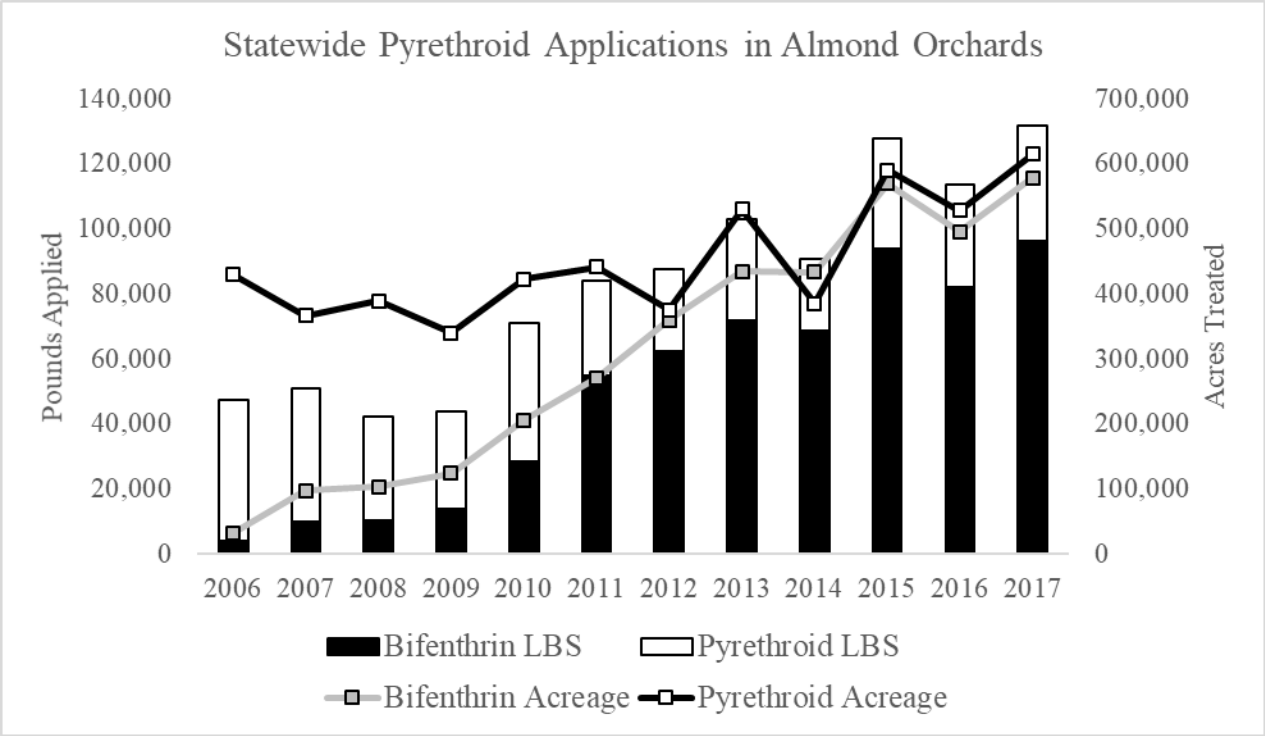


Figure 3.7. Bifenthrin use in Kern and Madera County almond orchards versus all other pyrethroids reported in the DPR pesticide use records (cyfluthrin, beta-cyfluthrin, (S)-cypermethrin, deltamethrin, esfenvalerate, fenpropathrin, lambda-cyhalothrin, gamma-cyhalothrin, permethrin) from 2006 to 2017.

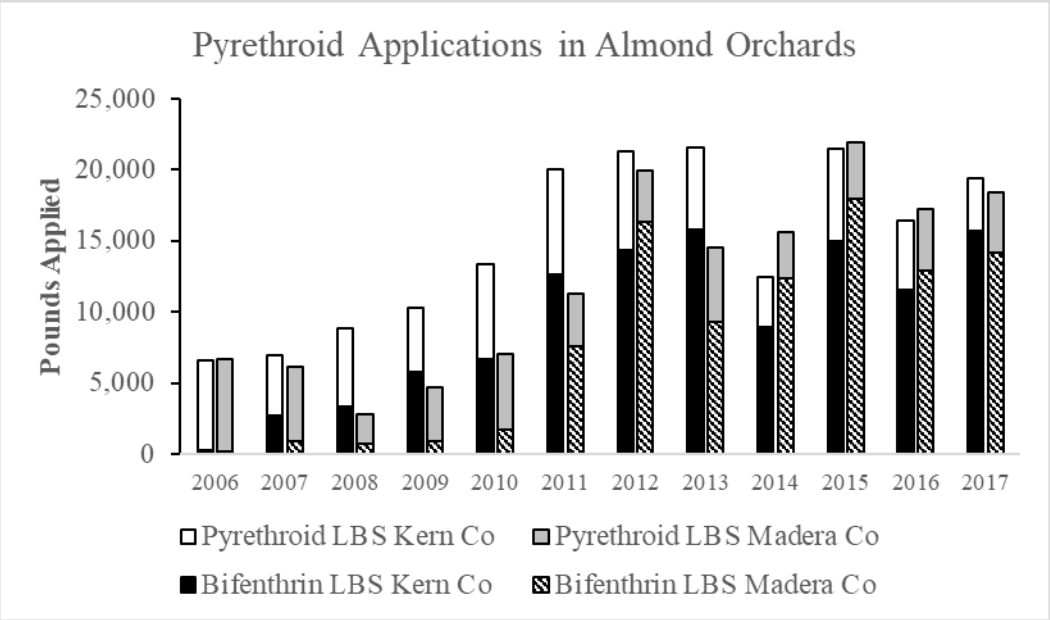
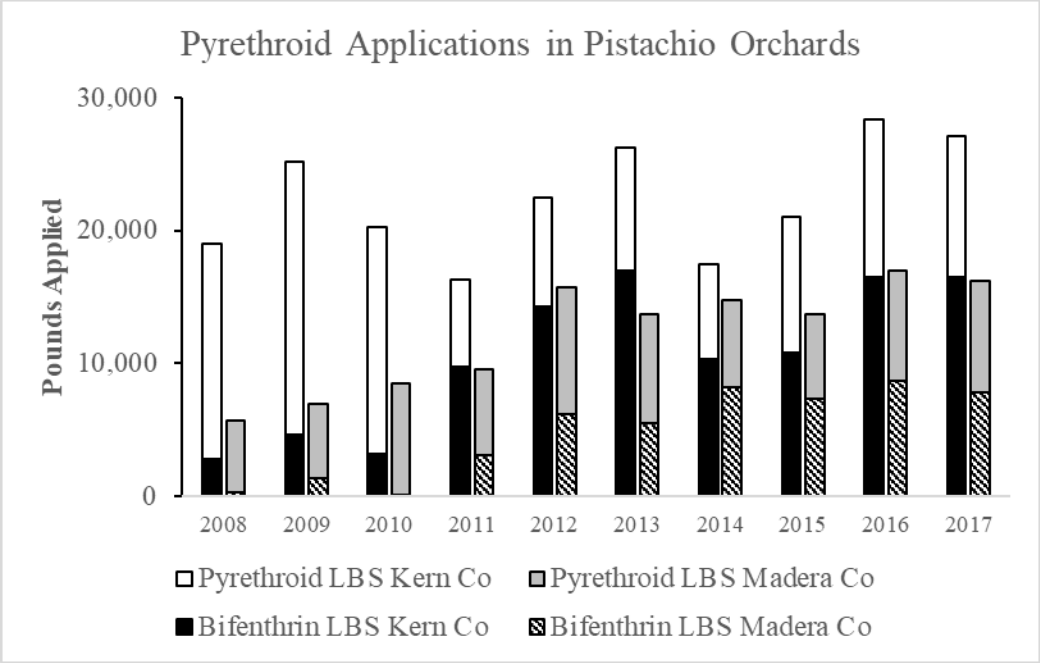


Figure 3.8. Bifenthrin use in Kern and Madera County pistachio orchards versus all other pyrethroids reported in the DPR pesticide use records (cyfluthrin, beta-cyfluthrin, (S)-cypermethrin, deltamethrin, fenpropathrin, lambda-cyhalothrin, permethrin) from 2008 to 2017.



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CHAPTER 4

IMPACT OF AGRICULTURAL ADJUVANTS ON THE TOXICITY OF THE DIAMIDE INSECTICIDES CHLORANTRANILIPROLE AND FLUBENDIAMIDE ON DIFFERENT LIFE STAGES OF THE NAVEL ORANGEWORM (*AMYELOIS TRANSITELLA*)

INTRODUCTION[1]

In California orchards, the navel orangeworm, *Amyelois transitella* Walker (Lepidoptera: Pyralidae), is the primary pest of almonds and pistachios as well as a serious pest of walnuts, figs, and pomegranates (Zalom *et al.* 2012). Neonates cause direct damage when they tunnel into nuts during hull-split and consume the nutmeat and generate large quantities of frass and webbing (Bentley *et al.* 2016). Damage by *A. transitella* also leaves nuts susceptible to infection by *Aspergillus flavus* and *Aspergillus parasiticus*, fungi that produce aflatoxins and contribute to further losses due to contamination (Palumbo *et al.* 2014). There are more than 687,000 hectares (>1.7 million ac) of almonds, pistachios, and walnuts in California, and *A. transitella* is currently managed by a combination of cultural control (removal of unharvested fruits, Higbee and Siegel 2009), insecticides, and, more recently, mating disruption. The insecticides used to control *A. transitella* are applied in rotation after the nut hulls split open (Higbee and Siegel 2012) and primarily target ingestion to arrest feeding damage. Insecticides for *A. transitella* control are typically applied one to three times based on the nut phenology of each variety (Higbee and

[1] This article is reprinted under Springer Nature Licenses which confer right to the Author(s) to use the substance of the Article in his/her future works, provided that the authors uses the final accepted manuscript and not the published version. This chapter appeared in Demkovich MR, Siegel JP, Walse SS, Berenbaum MR (2018) Impact of agricultural adjuvants on the toxicity of the diamide insecticides chlorantraniliprole and flubendiamide on different life stages of the navel orangeworm (*Amyelois transitella*). J Pest Sci 91: 1127-1136. DOI: https://doi.org/10.1007/s10340-018-0959-z. Journal of Pest Science: https://link.springer.com/journal/10340.

Siegel 2012, Zalom and Nicola 2014, Hamby *et al.* 2015) and include representatives from the organophosphate (IRAC Group 1B), pyrethroid (IRAC Group 3A), diacyl hydrazine (IRAC Group 18), diamide (IRAC Group 28), and spinosyn (IRAC Group 5) classes (CDPR 2015, Demkovich *et al.* 2015). The diamide chlorantraniliprole (Altacor®) is currently one of the top five most heavily applied insecticides in both almond and pistachio orchards (CDPR 2015), and although another diamide (flubendiamide, Belt®) was banned in 2016 by the United States Environmental Protection Agency, growers are still permitted to apply any existing stocks for treatment of *A. transitella* and other pests in orchards.

Damage due to *A. transitella* in tree nuts has increased during the past six years as a result of a combination of changes in the population dynamics in response to increased host availability, increased heat unit accumulation during the growing season, and increased crop value (>$49,000/ha some years) (J. Siegel, unpublished). Management is extremely challenging because of the ability of *A. transitella* to develop on multiple hosts grown in close proximity to almonds and pistachios, as well as to move between nut orchards, which in turn requires a strategy to manage immigrating pests as well as internal populations (Higbee and Siegel 2012, Bentley *et al.* 2016). Management is further complicated by the challenge of resistance management; because of the proximity of these different crops, there is a greater likelihood that consecutive generations are exposed to the same classes of insecticide. *A. transitella* may be exposed to five or more classes of insecticide in a growing season (Niu *et al.* 2012, Demkovich *et al.* 2015, Bagchi *et al.* 2016). The long-term impact of an insecticide on population dynamics is difficult to predict because life stages may vary in susceptibility and because sublethal effects can influence population dynamics. Although sublethal effects may reduce the rate that a population increases, they may not protect an individual nut from feeding damage.

Consequently, effective control may depend on the sum of a toxicant's effects across all life stages as well as the generation of *A. transitella* treated.

Adjuvant choice is an additional factor that can affect insecticide efficacy. Although formulated insecticides are water-soluble, they are combined with adjuvants to modify the physical properties of the spray mixtures (Acheampong and Stark 2004). Adjuvants are frequently paired with insecticides to enhance efficacy and include stickers, spreaders, wetting agents, emulsifiers, penetrators, foam suppressants, and dispersing agents (Mangan and Moreno 2001, Stark and Walthall 2003). However, adjuvants are regulated as additives under the Federal Insecticide, Fungicide, and Rodenticide Act (1996), and because they are not considered pesticides, toxicity data are not required by the Environmental Protection Agency (Stark and Walthall 2003). Thus, the effects of adjuvants on insecticide toxicity are not well known. In order to improve *A. transitella* control, it may be necessary to identify the pest life stages that are most vulnerable to an insecticide, as well as the adjuvant most likely to maximize these effects. We report here the results of a series of laboratory studies of five adjuvants in the spreader-sticker, spreader-penetrator, and wetter-spreader classes, alone and in combination with two diamide insecticides, chlorantraniliprole and flubendiamide (which was on the market when these studies commenced), targeting *A. transitella* adults and eggs. We also report confirmatory data on adjuvant differences from field trials and discuss the implications of our findings for improved control.

MATERIALS AND METHODS

Chemicals

Analytical standards of chlorantraniliprole (99.7 % purity, LOT: D100855-050) and flubendiamide (98.4% purity, LOT: 8064X) were supplied by DuPont (Wilmington, DE, USA) and Sigma Aldrich Co. (St. Louis, MO, USA), respectively. An analytical standard of bifenthrin (99.0 % purity, LOT: 1593700) was from Chem Service (West Chester, PA, USA). Acetonitrile, isopropyl alcohol, methanol and hexane were HPLC grade and obtained from Fisher (Pittsburg, PA). Aqueous solutions were prepared with deionized water (18 MΩ resistivity), unless otherwise noted. The classification, suggested rate, source, and names of the adjuvants used in our study are reported in Table 4.1. The adjuvant concentrations selected were within label rates and applied as follows: Cohere® at 226.8 g/378.5 l (8 oz/100 gal), Dyne-Amic® at 226.8 g/378.5 l (8 oz/100 gal), FastStrike® at 1,814.4 g/378.5 l (64 oz /100 gals), Induce® at 226.8 g/378.5 l (8 oz /100 gal), Latron B-1956® at 99.2 g /378.5 l (3.5 oz/100 gal). Dyne-Amic was applied at a concentration listed within the maximum label rate (1,792 g/378.5 l; 64 oz /100 gal) and also at a concentration below the recommended rate (226.8 g /378.5 l; 8 oz /100 gal), referred to as the low concentration, which is more indicative of current application rates used by almond and pistachio growers (J. Siegel, personal observation). Stock solutions of insecticides and adjuvants were prepared in methanolic (60% v/v) aqueous solutions at room temperature (22-23°C). Stock solutions of insecticides were prepared at 250 ppm, and subsequently diluted to 125 ppm with the same carrier. These insecticide concentrations correspond to approximately 116.9 g AI/ha (1.7 oz AI/ac). All insecticide stock solutions were stored at 4ºC. Adjuvants were stored at room temperature. This 125 ppm dose approximates the active ingredient applied to an acre (308.8 ppm/ha).

Spray Chamber Residues

A 30.5-cm diameter platform rotated at 36 rpm within an enclosed cylindrical spray chamber. The 50-mL reservoir was loaded with 10 mL of insecticide and/or adjuvant stock solutions to generally match field residue levels (*vide supra*). Spray solutions were delivered with 30 psi of nitrogen gas through a full cone fog nozzle (Spraying Systems, TG 0.4) positioned 61 cm above the center of the platform. The pyrethroid insecticide bifenthrin served as a proxy for estimating surface area coverage following sprays. A total of eight cellulose filter papers (55 mm, Whatman #1, GE Healthcare Life Sciences, Pittsburgh, PA) were localized radially 13 cm from center on the platform and a 500 ppm solution of bifenthrin was sprayed, on five replicate occasions, to yield 4.02 $\mu g/cm^2$. Deposition on filter paper represents 15.28% of the original solution; however, the collective surface area of the filter paper only represents 26% of the platform. Although recovery is unlikely uniform across the platform, we can estimate total recovery or surface area coverage on the platform as 58.66%. Bifenthrin residue was quantified with a Hewlett Packard 7890 gas chromatograph and a 5975 quadrupole mass spectrometer (GC-MS) operated with negative (-) chemical ionization (NCI) and methane reagent gas. Cool on-column injections (1 mL) were at 60°C with He carrier gas (8.2317psi). The oven program was heated from 60°C to 150°Cat 5°C/min, isothermal for 0 min, heated at 10°C/min to 270°C, and then isothermal for 5 min, , heated at 6°C/min to 300°C, and then isothermal for 0 min. Agilent ultra inert press fit universal unions fused three columns in series; a deactivated column (L = 8 cm, ID = 0.53 mm) onto which the injection were deposited, a retention-gap column (L = 2 m, ID = 0.25 mm) and a DB-5MS column (L = 30 m, ID = 0.25mm, Agilent Technologies, #122-5532). Transfer-line temperatures was 280°C. Analyte Rt, ((min) ± SE): bifenthrin, 30.77 ± 0.73 min. GC-NCIMS spectra were acquired for qualitative verification: Full scan spectra (m/z 100 to

500) were acquired at 0.54 s per scan for qualitative verification: m/z (% rel. inten.) Bifenthrin, m/z 126.9 (9), 174.8 (1), 190 (5), 205 (17), 241 (22), 366.8 (0.5), 386.1 (45), 401 (0.5). For insect assays, 5 bags containing three adult males and five bags containing three adult females were placed randomly near the perimeter on the revolving base for each application. Assays were replicated at least four times per treatment, including controls. For egg application, egg-strips were contained within a 100 x 15 mm Falcon petri dish (Corning, Tewksbury, MA) in the center of the base.

Colony and Preparation of Life Stages Tested

All *A. transitella* used in this study came from a laboratory colony designated CPQ that contained individuals recovered from almonds and reared in an incubator at 30°C, 16:8 photoperiod (Siegel *et al.* 2010) using a modified wheat bran diet (Finney and Brinkman 1967). For the egg studies, paper towels containing eggs collected one day after oviposition were cut into strips containing 50 viable (orange) eggs each. Any remaining eggs still white in color (recently laid) on the strip were punctured with a pin in order to ensure that the count was accurate. Immediately after adjuvant or insecticide exposure, egg strips were placed on Petri dishes filled with modified wheat bran diet. The Petri dishes were incubated at 30°C for 4 d as described and the unhatched eggs were counted. The Petri plates were returned to the incubator for another 14-16 d and the surviving larvae were counted. For the adult studies, fifth instar larvae were separated by sex and placed into separate emergence jars and allowed to pupate. Newly emerged adults were placed in bags made from fiberglass screen, three of the same sex per bag (Kuenen and Siegel 2016), and then sprayed. Adults were examined and scored at 24 h, 48 h, and 72 h intervals in the same manner for both laboratory and field trials.

Adult Assessment

In order to evaluate adult toxicity, the outcome variable of interest must be defined. Although there is an obvious distinction between dead and living moths, living moths fall into two categories. The first category is the moths that are unaffected by the toxicant, and adults that were flying inside the bag or moving freely were scored as living. The second category of living adults includes the moths that are affected but still alive (twitching intermittently or wing-fanning rapidly); we considered these moths to be moribund. We combined dead and moribund adults into a single category labeled "incapacity". For all studies, after the insecticide was applied, the screen bags were placed in large paper bags, which were then sealed with paper clips and kept at room temperature.

Field Application Adult Toxicity

The toxicity of Altacor (chlorantraniliprole) to *A. transitella* adults was assessed at the DuPont research farm in Madera County, using a dose of 126 g per 378.54 l (4.5 oz/ac) with either Latron B-1956 at 89.6 g/378.54 1 or Dyne-Amic at 336 g/378.54 l (3.2 oz/100 gal, 12 oz/100 gal, respectively) applied by DuPont personnel with an air-blast orchard sprayer. Belt was assessed at the Bayer Crop Science research farm in Fresno County using a dose of 112 g/378.54 l (4 oz/ac) with either Latron B-1956 or Dyne-Amic at the doses listed above, applied by Bayer personnel with an air-blast orchard sprayer. In these studies, the screen bags were placed in the center canopy at a height of 1.5-1.8 m and removed 24 h after the spray. Control adults were placed in an adjacent orchard and sprayed with water from an airblast orchard sprayer at the same volume as the treated adults. The control and treated adults were removed the next day and observed as described above.

Field Application Contact Toxicity Assay

Filter paper was hung in the canopy at 1.5-1.8 m and treated with air-blast orchard sprayers at the two research farms, using the same concentrations of insecticide and adjuvant for the adult trials. After 24 h in the field, the filter paper was brought back to the laboratory and placed in the middle of Petri dishes filled with modified wheat bran diet. An egg paper containing 50 eggs was placed in the center of the filter paper, such that neonates were forced to crawl over the filter paper to reach the diet. The Petri dishes were incubated for 21 d, after which the number of surviving larvae was recorded. Any larvae that lagged the rest of their cohort by two stadia were scored as "stunted".

Statistical Analysis

Chi-square analysis was used to determine if the data for males and female adults could be pooled; the pooled data are reported here. This technique was also used whenever two treatments were compared. Differences among adjuvants were analyzed by multiple regression with dummy coding (Cohen and Cohen 1983) employing JMP (v. 12, SAS Institute, Cary, NC). In this analysis, one treatment is used as the baseline (the intercept in the multiple regression equation and its value is the percentage killed) and all other treatments are compared to the baseline value (their estimate is added or subtracted from the intercept to determine percentage killed) to determine if their deviation is significant (determined by t-ratio). Two runs were conducted for each data set in order to group the treatments. The first run determined which treatments differed from methanol or the insecticide dissolved in methanol, and the second run distinguished among the remaining treatments. The Analysis of Variance (ANOVA) values for the first run are reported.

RESULTS

Adult Exposure to Adjuvant

There was only one adjuvant, FastStrike, that was significantly ($P< 0.0001$) more toxic than the others at 48 h, Table 4.2 (df 6; 1,793, F = 5.267, $P < 0.0001$). When moribund adults were included in the analysis, the number affected doubled for low concentrations of Dyne-Amic and quadrupled for the high concentration. FastStrike had the greatest effect by incapacitating (dead + moribund) 27.9% of adults ($P = 0.0009$), but the high concentration of Dyne-Amic incapacitated 20.8% of the adults and differed from the remaining adjuvants ($P < 0.02$).

Egg Exposure to Adjuvant

The base level of egg mortality in the controls was 1.7% and the egg mortality for the adjuvant treatments differed from this baseline (df 7; 14,742, F = 68.884, $P < 0.0001$). All treatments significantly increased mortality 5.6-12.6 fold above the background (Table 4.3). Cohere was the most toxic ($P < 0.0001$), causing mortality of 21.4%. The other treatments fell into two groups. FastStrike and the low concentration of Dyne-Amic were the least toxic and the high concentration of Dyne-Amic, Induce, methanol and Latron B-1956 were more toxic, ranging from 11.3 to 13.0%. When we followed the fate of the neonates from these exposed eggs, the baseline mortality for the neonates was 49.4%; the mortality from FastStrike, Induce and the two concentrations of Dyne-Amic were similar to this value, while Latron B-1956 and Cohere were more toxic (df 7; 6,183, F = 8.177, $P < 0.0001$), causing mortality of 56.2 to 57.4%. When overall mortality (egg + neonate) was calculated, Cohere and Latron B-1956 were the most toxic, killing 62.9-65.6% of the *A. transitella*, (1.39 X the baseline). All of the other

treatments caused greater mortality than that observed in the unsprayed control treatments (df 7; 14,042, F = 20.641, $P < 0.0001$) 52.8-57.4% (mortality at 1.1 X the baseline).

The sublethal effect quantified in this study was stunted growth. The baseline level of stunting was 12.6%, (Table 4.4) and stunting levels across adjuvant treatments fell into four groups (df 7; 6,238, F = 150.87, $P < 0.0001$). The high concentration of Dyne-Amic caused the greatest percentage of stunting, 39.5% ($P < 0.0001$), roughly three times greater than the baseline. FastStrike, Latron B-1956, and Cohere caused 27.6 to 33.7% stunting, and Induce caused the least stunting at 18.65%, which was 1.5 X the baseline level.

Adult Exposure to Adjuvant + Insecticide

For chlorantraniliprole, adult mortality fell into two groups (df 6; 1,073, F = 9.697, $P < 0.0001$), with Latron B-1956 and methanol in one group and all remaining adjuvants in the other group (Table 4.5). When incapacity was calculated, all adjuvants had the same impact 48 h after adults were exposed to the chlorantraniliprole. In contrast, the results for flubendiamide were more variable and the adjuvants fell into several groups (df 6; 953, F = 6.246, $P < 0.0001$). FastStrike caused the highest mortality, 40.0% ($P < 0.0001$), the two concentrations of Dyne-Amic and Induce caused intermediate mortality, and effects of both Latron B-1956 and Cohere were indistinguishable from the methanol carrier. When incapacity was calculated, FastStrike was still the most effective adjuvant ($P<0.0001$), with 99.2% of the adults affected, while the methanol carrier had the least effect, 52.5% of the adults affected ($P<0.0001$). The effect of the other adjuvants was intermediate. When the data for chlorantraniliprole and flubendiamide are compared, with the exception of FastStrike, the other adjuvants had a greater effect on adults when combined with chlorantraniliprole.

Egg exposure to Adjuvant + Insecticide

When eggs were treated with chlorantraniliprole combined with adjuvants, the adjuvants fell into three groups (Table 4.6) (df 6; 7,243, F = 11.9, $P < 0.0001$). Both FastStrike and Cohere treatments were the most toxic to eggs, causing 73.3% mortality, Latron B-1956 and the low concentration of Dyne-Amic treatments were intermediate in causing mortality, and treatments with Induce, the high concentration of Dyne-Amic, and the methanol carrier were comparable in their impact, causing 60.8 to 63.3% mortality. Mortality in the surviving neonates fell into two groups (df 6; 2,502, F = 3.299, $P = 0.003$), with Induce and the low concentration of Dyne-Amic causing the lowest mortality, 87.9 to 90.8%. When overall mortality (egg + neonate) is assessed, the adjuvants fell into two groups (df 6; 7,243, F = 5.271, $P < 0.0001$). FastStrike, Cohere and the high concentration of Dyne-Amic caused the highest mortality, 98.2 to 98.5%, and the remaining adjuvant treatments were the same as methanol.

When flubendiamide was assessed, fewer eggs survived the high concentration of Dyne-Amic than survived after exposure to the other treatments (df 6; 7,093, F = 10.55, $P < 0.0001$). When neonate mortality was assessed, treatments fell into two groups (df 6; 3,600, F = 6.161, $P < 0.0001$) with methanol and FastStrike causing the highest mortality and Dyne-Amic, Latron B-1956, and Cohere causing the lowest mortality. Overall mortality (egg + neonate) fell into two groups (df 6; 7,093, F = 6.069, $P < 0.0001$) with FastStrike and Methanol as the least toxic 97.6-98.3% ($P<0.001$) and the remaining treatments clustering together.

The overall mortality in the presence of the two diamides was so high that we could not detect differences in stunting among the treatments. Stunting was similar for all adjuvants, and, with the unsprayed controls as a baseline, diamide + adjuvant increased stunting 5.5 X (Chi Square = 450.5, $P < 0.0001$).

Field Exposure of Adults and Contact Toxicity of Neonates

Two adjuvants were evaluated in the field trials, Dyne-Amic at 336 g/378.5 l (12 oz/100 gal) and Latron B-1956 at 89.6 g/378.5 l (3.2 oz/100 gal), Table 4.7. For Altacor, Latron B-1956 was more effective than Dyne-Amic, 56.9% mortality compared to 30.2% (Chi Square = 28.08, $P < 0.001$). When incapacity was calculated, 66.8% of the adults were affected by Latron B-1956 compared to 46.7% of the adults by Dyne-Amic, a 1.4-fold difference (Chi Square = 16.0, $P < 0.001$). There was a different pattern for adults exposed to flubendiamide. The relationship between the adjuvants reversed and there were twice as many dead adults with Dyne-Amic (Chi Square = 21.0, $P < 0.001$) than with Latron B-1956. When incapacity was calculated, 56.4% of the adults were affected by Dyne-Amic, compared to 34.3% by Latron B-1956, a 1.6-fold difference (Chi Square=15.4, $P < 0.001$). In the contact toxicity assay for Altacor, Dyne-Amic caused greater mortality than Latron B-1956 (75.2% compared to 72.6%, Chi Square = 5.3, $0.025 > P > 0.01$) (Table 4.8), but there was no difference between the two adjuvants for flubendiamide (74.9% combined mortality).

DISCUSSION

In our laboratory study of the adult activity of adjuvants alone, FastStrike was the most toxic and also produced the greatest incapacity. When chlorantraniliprole was applied, three adjuvants were as effective as FastStrike + chlorantraniliprole in causing directly mortality, and all were equally effective in incapacitating adults. In contrast, for flubendiamide, FastStrike remained the most toxic adjuvant and also caused the highest incapacity. While it may be more satisfying from a sales point of view for a product to produce rapid and extensive mortality, functionally, a product can be equally effective if all adults exposed are quickly incapacitated.

The relationships in the initial adjuvant screen without toxicant were more predictive for flubendiamide than for chlorantraniliprole, suggesting that other mechanisms come into play for chlorantraniliprole.

The results from the field study examining formulated product were different. Although Latron B-1956 was indistinguishable from two adjuvants and methanol in the laboratory assays, when added to Altacor it caused both higher adult mortality and incapacity than did Dyne-Amic. The mortality from this treatment was also higher in the field application than in the laboratory study, but fewer adults were incapacitated in the field application. For Belt, the effect of these two adjuvants was reversed. Dyne-Amic caused higher mortality than did Latron B-1956, and mortality from field exposure was also higher than in the laboratory, but fewer adults were incapacitated. Despite these differences, we conclude that mortality is influenced by adjuvant choice, and the adult activity of flubendiamide is more variable than the adult activity of chlorantraniliprole.

Determining the impact of either adjuvant or insecticide on eggs is more challenging than calculating the impact on adults because sublethal effects must be assessed and the timeframe is longer. We measured one effect, stunting, but there may be other physiological responses influenced by diamide exposure, including increased mortality in late instar larvae, decreased adult emergence, and reduced adult fitness (Han *et al.* 2012; Sial and Brunner 2012). Although pooling egg and neonate mortality into a category called ovi-larvicidal activity may seem simpler, distinguishing between mortality in these two life stages is valuable because egg death effectively eliminates feeding damage. The high background mortality in our assay system, approaching 50%, occurs because *A. transitella* neonates under high-density laboratory conditions cannibalize conspecific eggs (Bush *et al.* 2017). Neonates may also consume or

otherwise inflict damage on conspecific larvae before beginning to consume the semi-synthetic diet (Siegel unpublished). The end result of the high baseline mortality is that identifying small differences among adjuvants is difficult.

Although adjuvants at the concentrations tested were intrinsically toxic to eggs, their activity was boosted by the diamides and the relationships among the adjuvants changed. In the initial screen, Cohere® was the most toxic to eggs while FastStrike was the least, but once chlorantraniliprole was added, Cohere and FastStrike were equally toxic to eggs. The addition of chlorantraniliprole essentially doubled egg mortality compared to the adjuvant alone and roughly doubled overall mortality. For both diamides, the high concentration of Dyne-Amic killed fewer eggs than other treatments but still produced high overall mortality. Perhaps it was less effective at penetrating the chorion in sufficient quantity to directly kill the egg, but exposure to Dyne-Amic was still sufficient to cause high neonate mortality by an undetermined mechanism. For the diamides, although there were significant differences in egg toxicity when they were combined with adjuvants, these differences vanished when overall mortality was calculated. However, from a grower perspective, adjuvants that cause the highest egg mortality may be preferred if they reduce feeding damage. Our results indicate that adjuvants such as Cohere, or others in that class, should be investigated further to assess their ovicidal activity when combined with insecticides belonging to other groups.

The contact toxicity assay differs from the laboratory assays because neonates emerge without exposure to the toxicant. Contact occurs when neonates crawl over the treated surface, but some may probe the filter paper and ingest insecticide. Our results are in agreement with the adult field study because adjuvant choice affected toxicity, and the magnitude of the effect depended on the insecticide used.

There are several explanations for the observed variability between laboratory and field assays. First, unformulated and formulated toxicants, alone and in combination with adjuvants, may differ in their activity. Second, laboratory application provides a level of control that does not occur in the field. Our coverage of at least 15.28% in the laboratory is three to seven times greater than the best coverage measured in almonds and pistachio orchards (J. Siegel, unpublished) and insecticide deposition decreases over height (Markle *et al.* 2016). Problems in spray coverage are not confined to tree crops and Pimentel (1995) reported that as little as 0.1% of a pesticide might reach its target in the field. Consequently, while our laboratory studies reveal a potential adjuvant impact on diamides, this effect may not be achieved in the field due to problems in coverage. However, the differences among adjuvants that we have identified warrant further exploration, especially if adjuvant choice can improve the toxicity of Altacor to *A. transitella*. Unfortunately, due to inherent limitations of both bioassays and our knowledge of *A. transitella* detoxification systems, we cannot predict how the adjuvants tested, or classes of adjuvant, will interact with other classes of insecticides used in almonds and pistachios, such as pyrethroids, diacylhydrazines, and spinosyns.

In conclusion, our study demonstrated that at least some adjuvants can be toxic to *A. transitella* and provides evidence that they can be used to optimize the activity of diamides to different life stages, although the mechanism of mortality is unknown. We corroborated the reports of previous researchers that adjuvants may be intrinsically toxic to a particular arthropod species and/or can synergize insecticides (Purcell *et al.* 1996 for tephritid flies, Cowles *et al.* 2000 for twospotted spider mite, *Tetranychus urticae* Koch, Srinivasan *et al.* 2008 for Asian citrus psyllid *Diaphorina citri* Kuwayama). In our laboratory assays, adjuvants were intrinsically toxic to *A. transitella* eggs and caused mortality as high as 66%. Adjuvants combined with a

diamide could cause egg mortality as high as 73% and overall mortality (egg + neonate) could exceed 99%. Developing a strategy to maximize egg mortality may improve management of this insect. Moreover, because up to 100% of the adults exposed to the diamide-adjuvant combinations in the laboratory were incapacitated, exploring how the adult activity of Altacor can be boosted in the field with this approach may yield new management strategies. If increasing the egg toxicity of Altacor is of interest, then the most promising adjuvants we have identified, or others in the same family, should be assessed further. Finally, we hope that our results stimulate research on the interaction of adjuvants with other classes of insecticides used to control *A. transitella*, so that new chemical management strategies can be developed that incorporate effective insecticide-adjuvant combinations for field sprays.

Compliance with Ethical Standards

This study was funded by the Almond Board of California (grant ABC 16.ENT01 NOW) and the California Pistachio Research Board (grant 2016 CPRB 082844). Mark R. Demkovich declares that he has no conflict of interest. Joel P. Siegel declares that he has no conflict of interest. Spencer S. Walse declares that he has no conflict of interest. May R. Berenbaum declares that she has no conflict of interest. Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

TABLES

Table 4.1. Classification, label rates, and manufacturers for all insecticides and adjuvants used in laboratory and field assays. © Journal of Pest Science (https://link.springer.com/journal/10340).

Chemical	Classification	Ground Application Label Rate	Manufacturer
Chlorantraniliprole (Altacor®)	Anthranilic diamide (IRAC Group 28)	Altacor: 126 g/378.5 l (4.5 oz /ac)	DuPont
Flubendiamide (Belt SC®)	Pthalic acid diamide (IRAC Group 28)	Belt SC (112 g/378.5 l (4 oz /ac)	Bayer
Cohere®	Water dispersible Spreader-Sticker	224-448 g/378.5 l (8-16 oz/100 gal)	Helena Chemical
Latron B-1956®	Spreader-Sticker	84-168 g/378.5 l (3-6 oz/100 gal)	Loveland Products, Inc
Induce®	Nonionic low foam Wetter-Spreader	224-1,344 g/378.5 l (8-48 oz/100 gal)	Helena Chemical
	Methylated Seed Oil+Organosilicone based		
Dyne-Amic®	Nonionic Surfactant Spreader-Penetrator	1,344-2,240 g/378.5 l (48-80 oz/100 gal)	Helena Chemical
FastStrike®	Methylated Vegetable Oil Wetter-Spreader	1,344-2,240 g/378.5 l (48-80 oz/100 gal)	J.R. Simplot

Table 4.2. Effect of exposure to adjuvants on adults of navel orangeworm, *Amyelois transitella*. Adjuvants were applied in the absence of insecticides with a spray tower in a laboratory setting. Incapacity is calculated by adding dead and moribund adults. Means separated by letters differ at $P < 0.05$.

48 h Adult Mortality			
	n	*Dead %*	*Incapacity %*
Low Conc Dyne-Amic	240	5.8 **A**	12.9 **A**
High Conc Dyne-Amic	120	5.8 **A**	20.8 **B**
Methanol 60%	480	6.9 **A**	11.9 **A**
Induce	240	7.9 **A**	16.7 **A**
Latron B-1956	240	8.8 **A**	16.7 **A**
Cohere	240	10.0 **A**	16.3 **A**
FastStrike	240	17.9 **B**	27.9 **B**

Table 4.3. Effect of exposure to adjuvant on egg and larval mortality. Adjuvants were applied in the absence of insecticides with a spray tower in a laboratory setting. Means separated by letters differ at $P < 0.05$.

			3 Week Larval Mortality		
	n	*Egg Kill%*	*Neonates emerged*	*Dead %*	*Overall Mortality%*
No Spray	2,700	1.7 **A**	2654	48.6 **A**	49.4 **A**
Low Conc Dyne-Amic	1,450	9.5 **B**	1312	47.8 **A**	52.8 **B**
FastStrike	1,800	9.9 **B**	1622	47.6 **A**	52.8 **B**
High Conc Dyne-Amic	1,200	11.3**C**	1064	52.0 **A**	57.4 **B**
Induce	1,400	12.3 **C**	1228	49.4 **A**	55.6 **B**
60% Methanol Carrier	2,650	12.4 **C**	2321	48.5**A**	54.9 **B**
Latron B-1956	1,400	13.0 **C**	1218	57.4 **B**	62.9 **C**
Cohere	1,450	21.4 **D**	1140	56.2 **B**	65.6 **C**

Table 4.4. Sublethal effect of adjuvants at the egg stage in the absence of insecticides, assessed 3 weeks after exposure in laboratory experiments with a spray tower. Means separated by letters differ at $P < 0.05$.

	Neonates Survived	*Stunting %*
No Spray	1,365	12.6 **A**
Induce	622	18.7 **B**
Dyne-Amic (Low Conc)	685	20.7 **B**
Latron B-1956	519	27.6 **C**
FastStrike	850	31.3 **D**
60% Methanol	1,195	32.4 **D**
Cohere	499	33.7 **D**
Dyne-Amic (High Conc)	511	39.5 **E**

Table 4.5. Effect of exposure to adjuvant + diamide on adults of navel orangeworm, *Amyelois transitella*. Adjuvants and insecticides were applied with a spray tower in a laboratory setting. Incapacity is calculated by adding dead and moribund adults. Means separated letters differ at $P < 0.05$.

48 h Adult Mortality			
	n	*Dead %*	*Incapacity %*
Chlorantraniliprole	360	24.2 **A**	96.7 **A**
Chlorantraniliprole + Latron B-1956	120	24.2 **A**	99.2 **A**
Chlorantraniliprole + Cohere	120	36.7 **B**	100.0 **A**
Chlorantraniliprole + High Conc Dyne-Amic	120	40.8 **B**	100.0 **A**
Chlorantraniliprole + Low Conc Dyne-Amic	120	41.7 **B**	100.0 **A**
Chlorantraniliprole + Induce	120	49.2 **B**	100.0 **A**
Chlorantraniliprole + FastStrike	120	52.5 **B**	100.0 **A**
Flubendiamide + Cohere	120	14.2 **A**	70.8 **B**
Flubendiamide	240	15.8 **A**	52.5 **A**
Flubendiamide + Latron B-1956	120	16.7 **A**	81.7 **B**
Flubendiamide + Induce	120	22.5 **B**	75.0 **B**
Flubendiamide + Low Conc Dyne-Amic	120	25.0 **B**	80.8 **B**
Flubendiamide + High Conc Dyne-Amic	120	26.7 **B**	75.8 **B**
Flubendiamide + FastStrike	120	40.0 **C**	99.2 **C**

Table 4.6. Effect of exposure to adjuvant + diamide on egg and larval mortality of navel orangeworm, *Amyelois transitella*. Adjuvants and insecticides were applied with a spray tower in a laboratory setting. Means separated by letters differ at $P < 0.05$.

			3 Week Larval Mortality		
	n	*Egg Kill%*	*Neonates emerged*	*Dead %*	*Overall Mortality%*
Chlorantraniliprole	2,250	60.8 **A**	881	89.8**A**	96.0 **A**
Chlorantraniliprole + Induce	750	62.4 **A**	282	87.9 **A**	95.5 **A**
Chlorantraniliprole + High Conc Dyne-Amic	1,250	63.3 **A**	459	95.2 **B**	98.2 **B**
Chlorantraniliprole + Latron B-1956	750	66.7 **B**	250	92.0 **AB**	97.3 **A**
Chlorantraniliprole + Low Conc Dyne-Amic	750	68.1 **B**	239	90.8 **A**	97.1 **A**
Chlorantraniliprole + FastStrike	750	73.3 **C**	200	93.5 **B**	98.3 **B**
Chlorantramiliprole + Cohere	750	73.3 **C**	200	94.5 **B**	98.5 **B**
Flubendiamide + High Conc Dyne-Amic	1,200	40.0 **A**	720	98.3 **B**	99.0 **B**
Flubendiamide	2,250	48.5 **B**	1,158	95.3 **A**	97.6 **A**
Flubendiamide + Latron B-1956	700	50.7 **B**	345	98.6 **B**	99.3 **B**
Flubendiamide + Induce	750	51.1 **B**	367	99.2 **B**	99.6 **B**
Flubendiamide + Low Conc Dyne-Amic	700	52.1 **B**	335	98.9 **B**	99.5 **B**
Flubendiamide + Cohere	750	54.0 **B**	345	98.8 **B**	99.5 **B**
Flubendiamide + FastStrike	750	55.1 **B**	337	96.1 **A**	98.3 **A**

Table 4.7. 48 Hour adult toxicity of Altacor (chlorantraniliprole) and Belt (flubendiamide) applied with DyneAmic and Latron B-1956 as adjuvants in the field. Incapacity is calculated by adding dead and moribund adults. Means separated by letters differ at $P < 0.05$.

Treatment	*n*	*Dead %*	*Incapacity %*
Control (Altacor experiment)	253	13.0 **A**	13.4 **A**
Altacor + Dyne-Amic	169	30.2 **B**	46.8 **B**
Altacor + Latron B-1956	253	56.9 **C**	66.8 **C**
Control (Belt experiment)	176	23.9 **A**	23.9 **A**
Belt + DyneAmic	156	50.0 **B**	56.4 **C**
Belt + Latron B-1956	185	25.4 **A**	37.8 **B**

Table 4.8. Neonate contact toxicity of Altacor (chlorantraniliprole) and Belt (flubendiamide) applied with DyneAmic and Latron B-1956 as adjuvants in the field. Means for an insecticide separated by letters differ at $P < 0.05$.

Treatment	*n*	*Dead*	*Reduction*
Control (Altacor experiment)	3,000	1,333 **A** (44.4%)	
Altacor + Dyne-Amic	3,000	2,257 **C** (75.2%)	55.4%
Altacor + LatronB-1956	3,000	2,178 **B** (72.6%)	50.7%
Control (Belt experiment)	1,950	715 **A** (36.7%)	
Belt + DyneAmic	1,950	1,462 **B** (75.0%)	60.5%
Belt + LatronB-1956	2,000	1,496 **B** (74.8%)	60.2%

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APPENDIX A

FULL-LENGTH AMINO ACID SEQUENCES OF 64 CARBOXYLESTERASES ANNOTATED IN THE *A. TRANSITELLA* GENOME

>AtraACE1
MRVVLAALTALAARALAGPHEHRARHHAPEHPIHFPAPAPPQPYRGHGEAVRYNPELD
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VSMQYRVASLGFLFFDTPDVPGNAGLFDQIMALQWVKDNIGYFGGNPHNVTLFGESAG
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>AtraACE2
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>AtraAE11
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>AtraAE12
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>AtraAE13
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>AtraAE14
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>AtraAE15
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>AtraAE16
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>AtraAE17
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>AtraAE18
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>AtraAE19
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>AtraAE20
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>AtraAE21
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SKKCH

>AtraAE22
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>AtraAE23
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>AtraAE24
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>AtraAE25
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KER

>AtraAE26
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FAYPALRSLKLQKEAGNNQIYLYEYSFVDEGGYPSGYKNLMVNGANHCGQTMTILDWP
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>AtraAE27
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>AtraAE28
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>AtraAE29
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VPDQLLDESSWL

>AtraAE30
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HVL

>AtraAE31
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>AtraAE32
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>AtraAE33
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>AtraAE34
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>AtraAE35
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>AtraAE36
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>AtraAE37
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>AtraAE38
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IITHSKRCPQVQINEETIIEVIDDIDCLTLSVFVPRGAENASVLFHIYDGSFTSGSGNPSLYG
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>AtraAE39
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>AtraAE40
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>AtraAE41
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>AtraAE42
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>AtraAE43
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>AtraAE44
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>AtraAE45
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>AtraBE1
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>AtraBE2
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>AtraGli
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>AtraIE1
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>AtraIE2
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>AtraJHE1
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>AtraJHE2
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>AtraNLG1
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>AtraNLG2
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>AtraNLG3
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>AtraNLG4
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